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on

COLLAGEN-BINDING MOLECULES THAT SELECTIVELY HOME TO TUMOR  
VASCULATURE AND METHODS OF USING SAME

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COLLAGEN-BINDING MOLECULES THAT SELECTIVELY HOME TO TUMOR  
VASCULATURE AND METHODS OF USING SAME

This invention was made with government support under CA74238, CA82713 and CA30199 awarded by the  
5 National Cancer Institute and grant HD31636 awarded by the National Institute of Child Health and Development. The government has certain rights in this invention.

This application claims benefit of the filing date of U.S. Provisional Application No. 60/\_\_\_\_\_,  
10 (yet to be assigned) filed August 28, 2002, which was converted from U.S. Serial No. 10/233,153, and which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

15 The present invention relates generally to the fields of molecular medicine and cancer biology and, more specifically, to collagen-binding molecules that selectively home to tumor vasculature.

BACKGROUND INFORMATION

20 A major hurdle to advances in treating cancer is the relative lack of agents that can selectively target the cancer while sparing normal tissue. For example, radiation therapy and surgery, which generally are localized treatments, can cause substantial damage to  
25 normal tissue in the treatment field, resulting in

scarring and loss of normal tissue. Chemotherapy, in comparison, which generally is administered systemically, can cause substantial damage to organs such as the bone marrow, mucosae, skin and small intestine, which undergo  
5 rapid cell turnover and continuous cell division. As a result, undesirable side effects such as nausea, loss of hair and drop in blood cell count often occur when a cancer patient is treated intravenously with a chemotherapeutic drug. Such undesirable side effects can  
10 limit the amount of a drug that can be safely administered, thereby hampering survival rate and impacting the quality of patient life.

Thus, there is a need for new therapeutic strategies for selectively targeting tumors and reducing  
15 the side effects associated with systemic therapy. The present invention satisfies this need by providing molecules that selectively home to tumor vasculature and which are suitable for selectively targeting chemotherapeutic drugs, gene therapy vectors or other  
20 agents to the tumor vasculature. Related advantages also are provided.

#### SUMMARY OF THE INVENTION

The present invention provides an isolated peptide or peptidomimetic which has a length of less than  
25 100 residues and includes the amino acid sequence CREKA (SEQ ID NO: 1) or a peptidomimetic thereof. Such an isolated peptide or peptidomimetic can have, for example, a length of less than 50 residues or a length of less than 20 residues. In particular embodiments, the

invention provides a peptide that includes the amino acid sequence CREKA (SEQ ID NO: 1) and has a length of less than 20, 50 or 100 residues.

The present invention further provides a  
5 conjugate containing a therapeutic agent linked to a homing molecule that selectively homes to tumor vasculature and selectively binds collagen. In one embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to tumor  
10 vasculature and that selectively binds non-helical collagen. In another embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to breast tumor vasculature and that selectively binds collagen. In another embodiment, the invention  
15 provides a conjugate containing a homing molecule that selectively homes to tumor vasculature and that selectively binds collagen IV. In yet another embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to tumor  
20 vasculature and that selectively binds denatured collagen IV in preference to native collagen IV. In a further embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to tumor vasculature and that selectively binds the alpha 2  
25 chain of collagen IV. In yet a further embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to tumor vasculature and selectively binds collagen and which is not an antibody or antigen-binding fragment thereof.

A variety of homing molecules that selectively home to tumor vasculature are useful in the conjugates of the invention. Such homing molecules include, without limitation, homing peptides and peptidomimetics. In one embodiment, the homing peptide or peptidomimetic portion of the conjugate has a length of at most 200 residues. In another embodiment, the homing peptide or peptidomimetic portion of the conjugate has a length of at most 50 residues. In a further embodiment, a conjugate of the invention contains a homing peptide or peptidomimetic that includes the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. In another embodiment, a conjugate of the invention contains a homing peptide or peptidomimetic that includes the amino acid sequence CREKA (SEQ ID NO: 1) or a peptidomimetic thereof. In still a further embodiment, a conjugate of the invention contains a homing peptide that includes the amino acid sequence CREKA (SEQ ID NO: 1).

A variety of therapeutic agents are useful in the conjugates of the invention including, without limitation, cancer chemotherapeutic agents, cytotoxic agents, anti-angiogenic agents, polypeptides, nucleic acid molecules and small molecules. As non-limiting examples, the invention provides a conjugate containing a therapeutic agent linked to a homing molecule that selectively homes to tumor vasculature and selectively binds collagen, where the therapeutic agent is a cancer chemotherapeutic agent, cytotoxic agent, anti-angiogenic agent, polypeptide, nucleic acid molecule or small molecule. As further non-limiting examples, the

invention provides a conjugate containing a therapeutic agent linked to a homing peptide or peptidomimetic that selectively homes to tumor vasculature and selectively binds collagen, where the therapeutic agent is a cancer chemotherapeutic agent, cytotoxic agent, anti-angiogenic agent, polypeptide, nucleic acid molecule or small molecule. As additional non-limiting examples, the invention provides a conjugate that contains a therapeutic agent linked to a homing peptide or peptidomimetic thereof, that selectively homes to tumor vasculature and selectively binds collagen, where the nucleic acid molecule or small molecule. Also provided herein is a conjugate that contains a therapeutic agent linked to a homing peptide or peptidomimetic containing the amino acid sequence CREKA (SEQ ID NO: 1), or a conservative variant or derivative thereof, that selectively homes to tumor vasculature and selectively binds collagen, where the nucleic acid molecule or small molecule. Further provided herein is a conjugate containing a therapeutic agent linked to a homing peptide or peptidomimetic containing the amino acid sequence CREKA (SEQ ID NO: 1) that selectively homes to tumor vasculature and selectively binds collagen, where the therapeutic agent is a cancer chemotherapeutic agent, cytotoxic agent, anti-angiogenic agent, polypeptide, nucleic acid molecule or small molecule.

Any of these or other conjugates of the invention can optionally include a virus moiety such as a phage.

The present invention also provides a conjugate containing a therapeutic agent and at least two homing  
5 molecules that each selectively homes to tumor vasculature and selectively binds collagen. The two homing molecules can each independently contain, for example, the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof.

10 Further provided by the invention is a conjugate containing a therapeutic agent and at least ten homing molecules that each selectively homes to tumor vasculature and selectively binds collagen. The ten homing molecules can each independently contain, for  
15 example, the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. The invention also provides a conjugate containing a therapeutic agent and at least 100 homing molecules that each selectively homes to tumor vasculature and  
20 selectively binds collagen. The 100 homing molecules included in the conjugate can each independently contain, for example, the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. Any of the above conjugates of the invention containing  
25 multiple homing molecules can optionally include a virus moiety such as a phage.

The present invention further provides a method of directing a moiety to tumor vasculature in a subject by administering to the subject a conjugate which  
30 contains the moiety linked to a homing molecule that

selectively homes to tumor vasculature and selectively binds collagen, thereby directing the moiety to tumor vasculature. In one embodiment, the homing molecule selectively homes to tumor vasculature and selectively binds non-helical collagen. In another embodiment, a method of the invention is practiced with a homing molecule that selectively homes to breast tumor vasculature and selectively binds collagen. In yet another embodiment, a method of the invention is practiced with a homing molecule that selectively binds collagen IV. In a further embodiment, a method of the invention is practiced with a homing molecule that selectively homes to tumor vasculature and selectively binds denatured collagen IV in preference to native collagen IV. In another embodiment, a method of the invention is practiced with a homing molecule that selectively homes to tumor vasculature and selectively binds the alpha 2 chain of collagen IV. In yet a further embodiment, a method of the invention is practiced with a homing molecule that selectively binds or antigen-binding fragment thereof.

A variety of homing molecules can be useful in the methods of the invention for directing a moiety to tumor vasculature. Useful homing molecules include, yet are not limited to, homing peptides or peptidomimetics such as those including the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. In one embodiment, a method of the invention for directing a moiety to tumor vasculature



is practiced with a homing molecule that includes the amino acid sequence CREKA (SEQ ID NO: 1), or a peptidomimetic thereof. In a further embodiment, a method of the invention for directing a moiety to tumor vasculature is practiced with a homing molecule that includes the amino acid sequence CREKA (SEQ ID NO: 1). A variety of moieties can be targeted to tumor vasculature according to a method of the invention including, without limitation, therapeutic agents, detectable agents and phage. As non-limiting examples, therapeutic agents to be directed to tumor vasculature include cancer chemotherapeutic agents, cytotoxic agents, anti-angiogenic agents, polypeptides, nucleic acid molecules and small molecules.

Also provided herein is a method of imaging tumor vasculature in a subject by (a) administering to the subject a conjugate containing a detectable agent linked to a homing molecule that selectively homes to tumor vasculature and selectively binds collagen; and (b) detecting the conjugate, thereby imaging tumor vasculature. In one embodiment, a method of the invention is practiced with a homing molecule that selectively homes to tumor vasculature and selectively binds to non-helical collagen. In another embodiment, an imaging method of the invention is practiced with a homing molecule that selectively homes to tumor vasculature and selectively binds collagen and which is not an antibody or antigen-binding fragment thereof. In a further embodiment, a method of the invention is used to image breast tumor vasculature.

A variety of homing molecules can be useful in the imaging methods of the invention, including homing peptides and peptidomimetics such as homing peptides or peptidomimetics containing the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. In one embodiment, the invention provides an imaging method that relies on a homing peptide or peptidomimetic containing the amino acid sequence CREKA (SEQ ID NO: 1) or a peptidomimetic thereof. In another embodiment, the invention provides an imaging method that relies on a homing peptide containing the amino acid sequence CREKA (SEQ ID NO: 1). Detectable agents useful in the imaging methods of the invention encompass, yet are not limited to, fluorophores such as fluorescein and rhodamine and radionuclides such as indium-111, technetium-99, carbon-11 and carbon-13.

The present invention additionally provides a method of reducing the number of tumor vessels in a subject by administering to the subject a conjugate which includes a therapeutic agent linked to a homing molecule that selectively homes to tumor vasculature and selectively binds collagen, thereby reducing the number of tumor vessels in the subject. A method of the invention can be useful, for example, for reducing the number of breast tumor vessels. In one embodiment, a method of the invention for reducing the number of tumor vessels is practiced with a homing molecule that selectively homes to tumor vasculature and selectively binds non-helical collagen. In another embodiment, a method of the invention for reducing the number of tumor vessels is practiced with a homing molecule that

selectively homes to tumor vasculature and selectively binds collagen and which is not an antibody or antigen-binding fragment thereof. Homing molecules useful in the invention encompass, without limitation, 5 homing peptides and peptidomimetics such as those including the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. A method of the invention can be practiced, for example, with a homing peptide or peptidomimetic containing the 10 amino acid sequence CREKA (SEQ ID NO: 1) or a peptidomimetic thereof. A method of the invention also can be practiced, for example, with a homing peptide containing the amino acid sequence CREKA (SEQ ID NO: 1). In a method of the invention for reducing the number of 15 tumor vessels, a variety of therapeutic agents can be incorporated into the conjugate administered to the subject, including, for example, cancer chemotherapeutic agents, cytotoxic agents, and anti-angiogenic agents.

20 Also provided herein is a method of treating cancer in a subject by administering to the subject a conjugate which contains a therapeutic agent linked to a homing molecule that selectively homes to tumor vasculature and selectively binds collagen. As a 25 non-limiting example, a method of the invention for treating cancer can be useful for treating breast cancer. Homing molecules useful in the invention include those which selectively home to tumor vasculature and selectively bind non-helical collagen. Homing molecules 30 useful in the invention also include those which selectively home to tumor vasculature and selectively

bind collagen IV, and those which selectively home to tumor vasculature and selectively bind denatured collagen IV in preference to native collagen IV. Homing molecules useful in the invention further include those  
5 which selectively home to tumor vasculature and selectively bind the alpha 2 chain of collagen IV. In one embodiment, a method of the invention for treating cancer relies on a homing molecule that selectively homes to tumor vasculature and selectively binds collagen and  
10 which is not an antibody or antigen-binding fragment thereof.

A variety of homing molecules can be included in a conjugate useful for treating cancer according to a method of the invention. In one embodiment, a method of  
15 the invention is practiced with a conjugate that contains a homing peptide or peptidomimetic. In another embodiment, a method of the invention is practiced with a conjugate in which the peptide or peptidomimetic portion of the conjugate has a length of at most 200 residues.  
20 In another embodiment, a method of the invention is practiced with a conjugate in which the peptide or peptidomimetic portion of the conjugate has a length of at most 50 residues. In yet another embodiment, a method of the invention is practiced with a conjugate which  
25 contains a homing peptide or peptidomimetic that includes the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. In still another embodiment, a method of the invention is practiced with a conjugate containing a homing peptide or  
30 peptidomimetic that includes the amino acid sequence CREKA (SEQ ID NO: 1), or a peptidomimetic thereof. In a

further embodiment, a method of the invention is practiced with a conjugate containing a homing peptide which includes the amino acid sequence CREKA (SEQ ID NO: 1). A conjugate useful for treating cancer according to a method of the invention incorporates a therapeutic agent such as, without limitation, a cancer chemotherapeutic agent, cytotoxic agent, anti-angiogenic agent, polypeptide, nucleic acid molecule or small molecule and can optionally further include one or more additional components such as a phage or other viral moiety.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the specificity of CREKA (SEQ ID NO: 1) phage homing to breast tumors. (A) MMTV PyMT mice. (B) Mice bearing MDA-MB-435 xenografts. Phage preparations were injected into tumor-bearing mice and recovered from breast tumor or the indicated normal tissue. One of several representative experiments is shown, with the number of pfu recovered represented by black bars. "Tumor/CREKA" indicates phage recovery from breast tumors subsequent to co-injection of free CREKA (SEQ ID NO: 1) peptide. "Tumor/control peptide" indicates phage recovery from breast tumors subsequent to co-injection of free control peptide.

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Figure 2 shows localization of CREKA (SEQ ID NO: 1)-displaying phage in mouse breast tumors. Phage were detected 15 minutes after injection by staining with anti-T7 phage antibodies. (A) CREKA (SEQ ID NO: 1)-phage staining in MMTV PyMT mammary carcinomas; (B) CREKA (SEQ

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ID NO: 1)-phage staining in MDA-MB-435 xenograft tumors;  
 (C) Non-recombinant T7 phage staining in breast tumor  
 tissue; (D) Staining of breast tumor tissue with control  
 antibody; (E) CREKA (SEQ ID NO: 1)-phage staining in  
 5 brain; (F) CREKA (SEQ ID NO: 1)-phage staining in heart;  
 (G) CREKA (SEQ ID NO: 1)-phage staining in kidney; (H)  
 CREKA (SEQ ID NO: 1)-phage staining in liver.

Figure 3 shows localization of  
 fluorescein-labeled and rhodamine-labeled CREKA (SEQ ID  
 10 NO: 1) peptide in MMTV PyMT tumors. (A) FITC-CREKA (SEQ  
 ID NO: 1) fluorescence in MMTV PyMT tumors 15 minutes  
 after injection. (B) FITC-CREKA (SEQ ID NO: 1)  
 fluorescence in brain 15 minutes after injection.  
 (C) FITC-CREKA (SEQ ID NO: 1) fluorescence in liver 15  
 15 minutes after injection. (D) FITC-CREKA (SEQ ID NO: 1)  
 fluorescence in MMTV PyMT tumors two hours following  
 injection. Nuclei were counter-stained with DAPI, and  
 tumor vasculature was visualized by intravenous injection  
 of FITC-tomato lectin. (E) Tumor vasculature of MMTV  
 20 PyMT tumors visualized by intravenous injection of  
 FITC-tomato-lectin (F) Rhodamine-CREKA (SEQ ID NO: 1)  
 and FITC-tomato-lectin staining in MMTV PyMT mouse heart  
 tissue counter-stained with DAPI.

25 Figure 4 shows the results obtained by  
 screening for binding on immobilized CREKA (SEQ ID NO: 1)  
 peptide. (A) Phage recovery of twelve individual clones  
 on immobilized CREKA (SEQ ID NO: 1) plates. (B) Amino  
 acid sequence of the CREKA (SEQ ID NO: 1)-binding phage  
 30 displayed protein fragment Clone #3. The cDNA encodes  
 a 138 amino acid fragment (SEQ ID NO: 2) related to the

corresponding portion of the collagen IV alpha-2 chain (SEQ ID NO: 3).

Figure 5 shows specificity of CREKA (SEQ ID NO: 1) binding to collagen. (A) Inhibition of phage displaying the clone #3 collagen IV fragment (SEQ ID NO: 2) binding to immobilized CREKA (SEQ ID NO: 1) by soluble CREKA (SEQ ID NO: 1) peptide. The data shown are representative of three separate experiments. (B) Binding of phage displaying clone #3 (SEQ ID NO: 2) to immobilized CREKA (SEQ ID NO: 1) in the presence of fibronectin (100 µg/ml), anti-collagen IV antibody, or control antibody. (C) Phage displaying CREKA (SEQ ID NO: 1) binding to immobilized collagen I, II, IV and X in native and denatured states.

Figure 6 shows the distribution of FITC-CREKA (SEQ ID NO: 1) and collagen IV in tumor tissue. (A) Collagen IV staining of a tumor from a MMTV PyMT mouse injected with FITC-CREKA (SEQ ID NO: 1). (B) Collagen IV staining of a tumor from a mouse not injected with FITC-CREKA (SEQ ID NO: 1).

Figure 7 shows the amino acid sequence of the human collagen IV  $\alpha$ 2 chain (SEQ ID NO: 4). See, also, Genbank NM\_001846.

### DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed, in part, to the discovery of homing molecules which selectively home to tumor vasculature, for example, selectively home to the matrix of breast tumor vasculature. As disclosed herein, peptide CREKA (SEQ ID NO: 1) was identified by in vivo panning as selectively homing to breast tumor vasculature as compared to the vasculature of a variety of normal organs. As shown in Figure 1A, about 130 times more of the CREKA (SEQ ID NO: 1)-displaying phage than control T7 phage homed to breast tumor vasculature in MMTV PyMT mice; furthermore, the CREKA (SEQ ID NO: 1)-displaying phage did not home to most other non-tumor tissues, including normal brain, heart, kidney, lung, pancreas and breast tissue. The CREKA (SEQ ID NO: 1)-displaying phage also homed to the vasculature of MDA-MB-435 human breast cancer xenografts with a 20-fold specificity over non-recombinant phage (see Figure 1B). As further disclosed herein, tumor homing of the CREKA (SEQ ID NO: 1) phage was specific, since coinjection of free peptide SEQ ID NO: 1 inhibited phage recovery from breast tumor tissue (Example 1 and Figure 1B).

As further disclosed herein in Example 2, immunoperoxidase staining showed that CREKA (SEQ ID NO: 1)-displaying phage were present in MMTV PyMT tumors (Figure 2A) and MDA-MB-435 xenografts (Figure 2B) fifteen minutes after intravenous injection of the phage while similarly injected non-recombinant T7 phage were undetectable in the tumors (Figure 2C). No CREKA (SEQ ID



NO: 1)-displaying phage were detected in control normal organs such as the brain, kidney and heart (Figures 2E, 2F and 2G, respectively). Although normal liver stained positive for the CREKA (SEQ ID NO: 1)-phage (Figure 2H), control T7 phage also localized to normal liver, indicating non-specific uptake by the reticuloendothelial system.

The results shown in Figure 3 demonstrate that fluorescein (FITC) and rhodamine-labeled CREKA (SEQ ID NO: 1) peptides also localized to MMTV PyMT tumors fifteen minutes after intravenous injection but were not present in normal tissues such as the brain or liver (compare Figure 3A with Figure 3B and 3C). The peptides were found primarily in the tumor periphery fifteen minutes after injection but could be detected in the entire tumor after two hours, localizing outside the blood vessels as shown in Figure 3D. The localization of CREKA (SEQ ID NO: 1) peptide to breast tumor vessels was confirmed by injection of rhodamine-labeled CREKA (SEQ ID NO: 1) peptide with FITC-tomato-lectin, which stains blood vessels, in MMTV PyMT mice (see Figure 3E). These results demonstrate that CREKA (SEQ ID NO: 1)-displaying phage and labeled CREKA (SEQ ID NO: 1) peptides quickly localize to the vasculature of human or murine breast tumors in preference to the vasculature of normal organs.

The present invention further is directed to the surprising discovery that a collagen such as collagen IV can serve as a receptor for the CREKA (SEQ ID NO: 1) peptide in the matrix of tumor vasculature. As disclosed herein in Example 3, a murine breast cancer

cDNA library was screened against immobilized CREKA (SEQ ID NO: 1) peptide. One clone (#3) bound avidly to the CREKA (SEQ ID NO: 1)-coated surface but not to an uncoated surface treated with the blocking buffer only (see Figure 4A). As shown in Figure 4B, this clone encodes a 138 amino acid fragment related to a portion of the collagen IV alpha-2 chain containing Gly-X-Y repeats, characteristic of the triple helical portion of collagen IV.

As further demonstrated in Figure 5A, the observed interaction between CREKA (SEQ ID NO: 1) and the collagen IV fragment of clone #3 (SEQ ID NO: 2) is specific, as it could be inhibited by an excess of cognate CREKA (SEQ ID NO: 1) peptide in a dose-dependent manner. Furthermore, as shown in Figure 5B, rabbit anti-mouse collagen IV serum, but not control serum, blocked the interaction between the phage-displayed collagen fragment and immobilized CREKA (SEQ ID NO: 1). In contrast, fibronectin, which binds to various collagens in their non-triple helical form, did not significantly reduce binding of CREKA (SEQ ID NO: 1)-displaying phage (Figure 5B), indicating that the collagen binding site for CREKA (SEQ ID NO: 1) is distinct from that of fibronectin.

As further disclosed herein in Example 3, CREKA (SEQ ID NO: 1)-displaying phage also were assayed for the ability to bind surfaces coated with several different collagens. Figure 5C shows that CREKA (SEQ ID NO: 1) phage bound to each of the collagens tested: collagens I, II, IV and X. Furthermore, denaturation of

the collagens by boiling enhanced the CREKA (SEQ ID NO: 1)-phage binding, indicating that non-helical collagen can be a receptor for CREKA (SEQ ID NO: 1) and other tumor homing molecules in tumor vasculature. These results were corroborated by the ability of gelatin, which is denatured collagen I, to completely block in vivo binding of CREKA (SEQ ID NO: 1) to collagen. The CREKA (SEQ ID NO: 1) peptide shares with fibronectin the ability to preferentially bind denatured collagen (Engvall et al., Cell 29:475-482 (1982)); however, as described above, fibronectin does not compete for CREKA (SEQ ID NO: 1) binding to collagen, indicating that CREKA (SEQ ID NO: 1) and fibronectin bind to different regions of denatured collagen.

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As additionally disclosed herein, localization of collagen IV was compared to the localization of CREKA (SEQ ID NO: 1) peptide in breast tumors. As shown in Figure 6, FITC-CREKA (SEQ ID NO: 1) partially co-localized with collagen IV in MMTV-PyMT tumors while a FITC-labeled control peptide did not, further supporting a role for collagen IV as a receptor for the CREKA (SEQ ID NO: 1) peptide in tumor vasculature. In sum, these results demonstrate that the CREKA (SEQ ID NO: 1) peptide binds a collagen IV  $\alpha$ -2 chain related protein and further indicate that collagen IV or a related collagen can act as a receptor for tumor homing molecules in tumor vasculature.

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Based on these findings, the present invention provides homing molecules and conjugates useful, for example, for directing a moiety to tumor vasculature, for

reducing the number of tumor vessels in a subject and for treating cancer. The conjugates of the invention also can be useful, for example, for imaging tumor vasculature such as breast tumor vasculature.

5                   Thus, the present invention provides an isolated peptide or peptidomimetic which has a length of less than 100 residues and which includes the amino acid sequence CREKA (SEQ ID NO: 1) or a peptidomimetic thereof. Such an isolated peptide or peptidomimetic can  
10 have, for example, a length of less than 50 residues or a length of less than 20 residues. In particular embodiments, the invention provides a peptide that includes the amino acid sequence CREKA (SEQ ID NO: 1) and has a length of less than 20, 50 or 100 residues.

15                   The peptides and peptidomimetics of the invention are provided in isolated form. As used herein in reference to a peptide or peptidomimetic of the invention, the term "isolated" means a peptide or peptidomimetic that is in a form that is relatively free  
20 from material such as contaminating polypeptides, lipids, nucleic acids and other cellular material that normally is associated with the peptide or peptidomimetic in a cell or that is associated with the peptide or peptidomimetic in a library or in a crude preparation.

25                   The peptides and peptidomimetics of the invention, including the bifunctional, multivalent and homing peptides and peptidomimetics discussed below, can have a variety of lengths. A peptide or peptidomimetic of the invention can have, for example, a relatively

short length of less than six, seven, eight, nine, ten, 12, 15, 20, 25, 30, 35 or 40 residues. A peptide or peptidomimetic of the invention also can be useful in the context of a significantly longer sequence. For example, 5 as disclosed herein, the CREKA peptide (SEQ ID NO: 1) maintained the ability to home when fused to a phage coat protein, confirming that a peptide of the invention can have selective homing activity when embedded in a larger protein sequence. Thus, a peptide or peptidomimetic of 10 the invention can have, for example, a length of up to 50, 100, 150, 200, 250, 300, 400, 500, 1000 or 2000 residues. In particular embodiments, a peptide or peptidomimetic of the invention has a length of at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or 200 15 residues. In further embodiments, a peptide or peptidomimetic of the invention has a length of 5 to 200 residues, 5 to 100 residues, 5 to 90 residues, 5 to 80 residues, 5 to 70 residues, 5 to 60 residues, 5 to 50 residues, 5 to 40 residues, 5 to 30 residues, 5 to 20 20 residues, 5 to 15 residues, 5 to 10 residues, 10 to 200 residues, 10 to 100 residues, 10 to 90 residues, 10 to 80 residues, 10 to 70 residues, 10 to 60 residues, 10 to 50 residues, 10 to 40 residues, 10 to 30 residues, 10 to 20 residues, 20 to 200 residues, 20 to 100 residues, 20 to 25 90 residues, 20 to 80 residues, 20 to 70 residues, 20 to 60 residues, 20 to 50 residues, 20 to 40 residues or 20 to 30 residues. As used herein, the term "residue" refers to an amino acid or amino acid analog.

The present invention also provides an isolated 30 peptide or peptidomimetic containing the amino acid sequence CREKA (SEQ ID NO: 1), or a conservative variant

or peptidomimetic of one of this sequence. As used herein in reference to a specified amino acid sequence, a "conservative variant" is a sequence in which a first amino acid is replaced by another amino acid or amino acid analog having at least one biochemical property similar to that of the first amino acid; similar properties include, for example, similar size, charge, hydrophobicity or hydrogen-bonding capacity.

As an example, a conservative variant can be a sequence in which a first uncharged polar amino acid is conservatively substituted with a second (non-identical) uncharged polar amino acid such as cysteine, serine, threonine, tyrosine, glycine, glutamine or asparagine or an analog thereof. A conservative variant also can be a sequence in which a first basic amino acid is conservatively substituted with a second basic amino acid such as arginine, lysine, histidine, 5-hydroxylysine, N-methyllysine or an analog thereof. Similarly, a conservative variant can be a sequence in which a first hydrophobic amino acid is conservatively substituted with a second hydrophobic amino acid such as alanine, valine, leucine, isoleucine, proline, methionine, phenylalanine or tryptophan or an analog thereof. In the same way, a conservative variant can be a sequence in which a first acidic amino acid is conservatively substituted with a second acidic amino acid such as aspartic acid or glutamic acid or an analog thereof; a sequence in which an aromatic amino acid such as phenylalanine is conservatively substituted with a second aromatic amino acid or amino acid analog, for example, tyrosine; or a sequence in which a first relatively small amino acid

such as alanine is substituted with a second relatively small amino acid or amino acid analog such as glycine or valine or an analog thereof. As non-limiting examples, conservative variants of CREKA (SEQ ID NO: 1) include

5 SREKA (SEQ ID NO: 5); CKEKA (SEQ ID NO: 6); CRDKA (SEQ ID NO: 7); CRERA (SEQ ID NO: 8); CREKV (SEQ ID NO: 9); SKEKA (SEQ ID NO: 10); SRDKA (SEQ ID NO: 11); SRERA (SEQ ID NO: 12); SREKV (SEQ ID NO: 13); CKDKA (SEQ ID NO: 14); CKERA (SEQ ID NO: 15); CKEKV (SEQ ID NO: 16); CRDRA (SEQ ID NO: 17); CRDKV (SEQ ID NO: 18); and CRRV (SEQ ID NO: 19). It is understood that conservative variants of CREKA (SEQ ID NO: 1) encompass sequences containing one, two, three, four or more amino acid substitutions relative to SEQ ID NO: 1 and that such variants can

15 include naturally and non-naturally occurring amino acid analogs.

The invention further provides a chimeric protein containing a peptide or peptidomimetic of the invention, or a homing peptide or peptidomimetic of the invention, fused to a heterologous protein. The

20 invention provides, for example, a chimeric protein containing a homing peptide or peptidomimetic that selectively homes to tumor vasculature and selectively binds collagen such as non-helical collagen or collagen IV. In one embodiment, the heterologous protein has a therapeutic activity such as cytokine activity, cytotoxic activity or pro-apoptotic activity. In a further embodiment, the heterologous protein is an antibody or antigen-binding fragment thereof. In other

30 embodiments, the chimeric protein includes a peptide or peptidomimetic containing the amino acid sequence CREKA

(SEQ ID NO: 1), or a conservative variant or peptidomimetic thereof, fused to a heterologous protein. The term "heterologous," as used herein in reference to a protein fused to a peptide or peptidomimetic of the invention, means a protein derived from a source other than the gene encoding the peptide of the invention or from which the peptidomimetic is derived. A chimeric protein of the invention can have a variety of lengths including, but not limited to, a length of less than 100 residues, less than 200 residues, less than 300 residues, less than 400 residues, less than 500 residues, less than 800 residues or less than 1000 residues.

The invention also provides a bifunctional peptide or peptidomimetic which contains a homing peptide or peptidomimetic that selectively homes to tumor vasculature and selectively binds collagen fused to a second peptide or peptidomimetic having a separate function. The collagen can be, for example, non-helical collagen or collagen IV. Such bifunctional peptides and peptidomimetics have at least two functions conferred by different portions of the full-length molecule and can, for example, display anti-angiogenic activity or pro-apoptotic activity in addition to selective homing activity. As an exemplary bifunctional peptide, the invention provides CREKA-GG-D(KLAKLAK)<sub>2</sub>. In such a bifunctional peptide, the CREKA (SEQ ID NO: 1) portion exhibits selective homing activity, while the D(KLAKLAK)<sub>2</sub> portion exhibits pro-apoptotic activity.

The present invention further provides an isolated multivalent peptide or peptidomimetic that



includes at least two subsequences each independently containing the amino acid sequence CREKA (SEQ ID NO: 1), or a conservative variant or peptidomimetic thereof. The multivalent peptide or peptidomimetic can have, for  
5 example, at least three, at least five or at least ten of such subsequences each independently containing the amino acid sequence CREKA (SEQ ID NO: 1), or a conservative variant or peptidomimetic thereof. In particular embodiments, the multivalent peptide or peptidomimetic  
10 has two, three, four, five, six, seven, eight, nine, ten, fifteen or twenty identical or non-identical subsequences containing the amino acid sequence CREKA (SEQ ID NO: 1), or a conservative variant or peptidomimetic thereof. In a further embodiment, the multivalent peptide or  
15 peptidomimetic contains identical subsequences, which consist of the amino acid sequence SEQ ID NO: 1, or a conservative variant or peptidomimetic thereof. In a further embodiment, the multivalent peptide or peptidomimetic contains contiguous identical or  
20 non-identical subsequences, which are not separated by any intervening amino acids. In yet further embodiments, the multivalent peptide or peptidomimetic is cyclic or otherwise conformationally constrained.

Thus, the invention provides peptides and  
25 peptidomimetics, including bifunctional and multivalent peptides and peptidomimetics, and homing peptides and peptidomimetics discussed further below. As used herein, the term "peptide" is used broadly to mean peptides, proteins, fragments of proteins and the like. The term  
30 "peptidomimetic," as used herein, means a peptide-like molecule that has the activity of the peptide upon which

it is structurally based. Such peptidomimetics include chemically modified peptides, peptide-like molecules containing non-naturally occurring amino acids, and peptoids and have an activity such as selective homing  
5 activity of the peptide upon which the peptidomimetic is derived (see, for example, Goodman and Ro, Peptidomimetics for Drug Design, in "Burger's Medicinal Chemistry and Drug Discovery" Vol. 1 (ed. M.E. Wolff; John Wiley & Sons 1995), pages 803-861).

10 A variety of peptidomimetics are known in the art including, for example, peptide-like molecules which contain a constrained amino acid, a non-peptide component that mimics peptide secondary structure, or an amide bond isostere. A peptidomimetic that contains a constrained,  
15 non-naturally occurring amino acid can include, for example, an  $\alpha$ -methylated amino acid;  $\alpha,\alpha$ -dialkylglycine or  $\alpha$ -aminocycloalkane carboxylic acid; an  $N^\alpha$ - $C^\alpha$  cyclized amino acid; an  $N^\alpha$ -methylated amino acid; a  $\beta$ - or  $\gamma$ -amino cycloalkane carboxylic acid; an  $\alpha,\beta$ -unsaturated amino  
20 acid; a  $\beta,\beta$ -dimethyl or  $\beta$ -methyl amino acid; a  $\beta$ -substituted-2,3-methano amino acid; an  $N$ - $C^\delta$  or  $C^\alpha$ - $C^\delta$  cyclized amino acid; a substituted proline or another amino acid mimetic. A peptidomimetic which mimics peptide secondary structure can contain, for example, a  
25 non-peptidic  $\beta$ -turn mimic;  $\gamma$ -turn mimic; mimic of  $\beta$ -sheet structure; or mimic of helical structure, each of which is well known in the art. A peptidomimetic also can be a peptide-like molecule which contains, for example, an amide bond isostere such as a retro-inverso modification;  
30 reduced amide bond; methylenethioether or methylene-sulfoxide bond; methylene ether bond; ethylene bond;

thioamide bond; trans-olefin or fluoroolefin bond;  
1,5-disubstituted tetrazole ring; ketomethylene or  
fluoroketomethylene bond or another amide isostere. One  
skilled in the art understands that these and other  
5 peptidomimetics are encompassed within the meaning of the  
term "peptidomimetic" as used herein.

Methods for identifying a peptidomimetic are  
well known in the art and include, for example, the  
screening of databases that contain libraries of  
10 potential peptidomimetics. As an example, the Cambridge  
Structural Database contains a collection of greater than  
300,000 compounds that have known crystal structures  
(Allen et al., Acta Crystallogr. Section B, 35:2331  
(1979)). This structural depository is continually  
15 updated as new crystal structures are determined and can  
be screened for compounds having suitable shapes, for  
example, the same shape as a peptide of the invention, as  
well as potential geometrical and chemical  
complementarity to a target molecule. Where no crystal  
20 structure of a peptide of the invention or a target  
molecule that binds the peptide is available, a structure  
can be generated using, for example, the program CONCORD  
(Rusinko et al., J. Chem. Inf. Comput. Sci. 29:251  
(1989)). Another database, the Available Chemicals  
25 Directory (Molecular Design Limited, Information Systems;  
San Leandro CA), contains about 100,000 compounds that  
are commercially available and also can be searched to  
identify potential peptidomimetics of a peptide of the  
invention, for example, with activity in selectively  
30 homing to tumor vasculature and selectively binding to  
collagen.

If desired, an isolated peptide or peptidomimetic of the invention, or a homing molecule of the invention as discussed further below, can be cyclic or otherwise conformationally constrained. As used herein, a "conformationally constrained" molecule, such as a peptide or peptidomimetic, is one in which the three-dimensional structure is maintained substantially in one spatial arrangement over time. Conformationally constrained molecules can have improved properties such as increased affinity, metabolic stability, membrane permeability or solubility. Methods of conformational constraint are well known in the art and include cyclization as discussed further below.

As used herein in reference to a peptide or peptidomimetic, the term "cyclic" means a structure including an intramolecular bond between two non-adjacent amino acids or amino acid analogues. The cyclization can be effected through a covalent or non-covalent bond. Intramolecular bonds include, but are not limited to, backbone to backbone, side-chain to backbone and side-chain to side-chain bonds. A preferred method of cyclization is through formation of a disulfide bond between the side-chains of non-adjacent amino acids or amino acid analogs. Residues capable of forming a disulfide bond include, for example, cysteine (Cys), penicillamine (Pen),  $\beta,\beta$ -pentamethylene cysteine (Pmc),  $\beta,\beta$ -pentamethylene- $\beta$ -mercaptopropionic acid (Pmp) and functional equivalents thereof.

A peptide or peptidomimetic also can cyclize, for example, via a lactam bond, which can utilize a side-chain group of one amino acid or analog thereof to form a covalent attachment to the N-terminal amine of the amino-terminal residue. Residues capable of forming a lactam bond include aspartic acid (Asp), glutamic acid (Glu), lysine (Lys), ornithine (Orn),  $\alpha,\beta$ -diaminopropionic acid,  $\gamma$ -amino-adipic acid (Adp) and M-(aminomethyl)benzoic acid (Mamb). Cyclization additionally can be effected, for example, through the formation of a lysinonorleucine bond between lysine (Lys) and leucine (Leu) residues or a dityrosine bond between two tyrosine (Tyr) residues. The skilled person understands that these and other bonds can be included in a cyclic peptide or peptidomimetic of the invention.

The present invention also provides a conjugate containing a therapeutic agent linked to a homing molecule that selectively homes to tumor vasculature and selectively binds collagen. In one embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to breast tumor vasculature and that selectively binds collagen. In another embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to tumor vasculature and that selectively binds non-helical collagen. In still another embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to tumor vasculature and that selectively binds collagen IV. In a further embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to tumor vasculature and

that selectively binds denatured collagen IV in preference to native collagen IV. In a further embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to tumor  
5 vasculature and that selectively binds the alpha 2 chain of collagen IV. In yet a further embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to tumor vasculature and selectively binds collagen and which is not an antibody  
10 or antigen-binding fragment thereof.

The invention further provides a conjugate containing a tumor homing peptide or other homing molecule that selectively homes to tumor vasculature, where the homing molecule selectively binds a polypeptide  
15 or fragment thereof containing Gly-X-Y repeats. In another embodiment, the invention provides a conjugate containing a tumor homing peptide or other homing molecule that selectively homes to tumor vasculature, where the homing molecule selectively binds collagen or a  
20 fragment thereof containing Gly-X-Y repeats. In a further embodiment, the invention provides a conjugate containing a tumor homing peptide or other homing molecule that selectively homes to tumor vasculature, where the homing molecule selectively binds collagen IV  
25 or a fragment thereof, such as a fragment containing Gly-X-Y repeats.

The present invention is directed, in part, to the discovery that a collagen IV alpha-2 chain related polypeptide can act as a receptor for the CREKA (SEQ ID  
30 NO: 1) tumor homing peptide. Collagens are a major

component of the extracellular matrix (ECM), an interconnected molecular network providing mechanical support for cells and tissues and regulating biochemical and cellular processes such as adhesion, migration, gene expression and differentiation (Timpl and Brown, Bioessays 18:123-132 (1995); Timpl, Curr. Opin. Cell Biol. 8:618-624 (1996); Herbst et al., J. Cell Biol. 106:1365-1373 (1998); Tsai, Trends Cell Biol. 8:192-195 (1998); and Dogic et al., Curr. Topics Pathol. 93:75-83 (1999)). In higher animals, at least 19 distinct collagen types differing in their higher order structures and functions have been identified based on the presence of the characteristic collagen triple-helix structure. In addition, at least 15 other proteins containing collagenous domains have been identified; such proteins include Clq and MSR (Hoppe and Reid, Prot. Sci. 3:1143-1158 (1994); and Myllyharju and Kivirikko, Ann. Med. 33:7 (2001)). The collagens are sometimes categorized into the fibrillar and nonfibrillar collagens. The fibrillar (interstitial) collagens include types I, II, III, V and XI, while the nonfibrillar collagens include types IV, VI, IX, X, XI, XII, XIV and XIII.

The amino acid sequence of collagen shows two unique features: (1) glycine is present as every third residue, generating a repeating (Gly-X-Y)<sub>n</sub> pattern and (2) a high proportion of residues (about 20%) are the imino acids proline and hydroxyproline (Brodsky and Ramshaw, Matrix Biol. 15:545-554 (1997)). See, for example, Figure 7, which shows the sequence of the human collagen type IV  $\alpha 2$  chain. The unique tertiary structure

common to collagens consists of three parallel left-handed polyproline II-type strands wound around a common axis to form a triple helix with a shallow right-handed superhelical pitch. The packing of the collagen coiled-coil structure requires that every third residue be glycine, resulting in the repeated Gly-X-Y sequence characteristic of the collagens. The residue in the X position of the triplets is often L-proline, and the residue in the Y position is often

10 4(R)-hydroxy-L-proline (Jenkins and Raines, Royal Soc. Chem. 19:49-59 (2002)).

Collagen IV is a major component of the vascular basement membrane, which is a specialized form of the extracellular matrix that separates epithelia from its underlying mesenchyme and lines blood vessels (Hudson et al., J. Biol. Chem. 268:26033-26036 (1993); Timpl and Brown, *supra*, 1995; Timpl, *supra*, 1996). Type IV collagen plays a role in the interaction of basement membranes with cells, either directly or mediated by laminin or laminin/nidogen binding, and also binds heparin and heparan sulfate proteoglycans (Marneros and Olsen, Matrix Biol. 20:337-345 (2001)).

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Six distinct genes encode the six type IV collagen chains denoted  $\alpha 1$ - $\alpha 6$ (IV). The most widely expressed form of collagen IV, also known as "basement membrane collagen," is composed of two  $\alpha 1$ (IV) chains and one  $\alpha 2$ (IV) chain and is found in the basement membrane of virtually all blood vessels, while the  $\alpha 3$ - $\alpha 6$ (IV) chains are found in specialized basement membranes such as the kidney glomerular basement membrane.  $\alpha 1$ (IV)<sub>2</sub> $\alpha 2$ (IV)

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trimers contain an "RGD" cell-binding site within the triple-helical domain; this site mediates binding by two integrin receptors:  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  (Messent et al., J. Cell Sci. 111:1127-1135 (1998); and Emsley et al.,  
 5 Cell 100:47-56 (2000)).

The individual chains of collagen type IV contain a cysteine-rich (7S) domain at the amino-terminus, a central triple-helical collagenous domain, and a carboxy-terminal non-collagenous (NC1)  
 10 domain. Type IV collagen molecules form a network structure through covalently cross-linked and laterally associated 7S domains, end-to-end interactions of the NC1 domains and lateral association of the central collagenous domains. The most highly conserved portions  
 15 of collagen IV are in the end regions: the globular NC1 domain at the carboxy-terminus, and the amino-terminal region of the triple helical domain. These evolutionarily conserved end regions have been shown to be important for the end-to-end aggregation of  
 20 collagen IV chains. In contrast, the triple-helical domain of collagen IV molecules shows higher sequence variability. Non-triple-helical segments frequently interrupt the triple-helical domain, with the incidence of non-Gly-X-Y repeat segments generally higher in the  
 25 amino-terminal half of the triple-helical domain than in the carboxy-terminal half.

The tumor homing molecules useful in the invention are characterized, in part, by the ability to selectively bind a collagen such as collagen IV. As used  
 30 herein, the term "collagen" means a polypeptide

containing Gly-X-Y repeats and that can form a triple helix of three parallel left-handed strands wound around a common axis to form a triple helix. Thus, the term collagen as used herein encompasses the individual  
5 component polypeptide chains that make up collagen as well as collagen in its native form. Collagens generally contain a high proportion of proline and hydroxyproline residues. A collagen can be a fibrillar or nonfibrillar collagen of any species, type or isoform in native,  
10 denatured or partially denatured form. Thus, a homing molecule of the invention can selectively bind, without limitation, a mammalian collagen such as a human, bovine, rat or mouse collagen; any of a variety of types of collagen such as collagen type I, type II, type IV or  
15 type X; a collagen which, in its native state, includes one or more non-helical portions; and any of a variety of collagen isoforms including a collagen containing one or more  $\alpha 2$  chains such as  $\alpha 2(\text{IV})$  chains, for example, the  $\alpha 1(\text{IV})_2\alpha 2(\text{IV})$  isoform of collagen type IV. The term  
20 collagen encompasses both native collagen as well as non-helical collagen. It is recognized that each of the conjugates and methods disclosed herein can be practiced, if desired, with a tumor homing molecule that selectively homes to tumor vasculature and selectively binds native  
25 collagen.

As used herein, the term "non-helical collagen" means a collagen that lacks triple helical structure in a portion or over the full-length of the molecule. Non-helical collagens include collagens which are partly  
30 or entirely non-helical such as, without limitation, native, wild type collagens containing non-helical

portions; wild type collagens which are partly or entirely non-helical due to cleavage, modification by a kinase, protease or other enzyme, or interaction with one or more proteins which alter their structure; mutant  
5 forms of collagen; random-coiled collagens; and denatured collagens.

The term "collagen IV," as used herein, refers to a collagen that exhibits greater sequence similarity to the human collagen IV sequence SEQ ID NO: 4 shown in  
10 Figure 7 than to another type of human collagen. A collagen IV can have, for example, greater than 40% amino acid identity with SEQ ID NO: 4, or can have, for example, greater than 50%, 60%, 70%, 80%, 90% or 95% identity with SEQ ID NO: 4.

15 As disclosed herein, CREKA (SEQ ID NO: 1) or another tumor homing molecule that selectively homes to tumor vasculature can bind collagen or a region or chain thereof and can exhibit enhanced binding to denatured or non-helical collagen as compared to native collagen. In  
20 one embodiment, the invention provides a conjugate containing CREKA (SEQ ID NO: 1) or another tumor homing molecule that selectively homes to tumor vasculature, where the homing molecule selectively binds a collagen or a region or chain thereof. In another embodiment, the  
25 invention provides a conjugate containing CREKA (SEQ ID NO: 1) or another tumor homing molecule that selectively homes to tumor vasculature, where the homing molecule selectively binds non-helical collagen or a non-helical region of a collagen. In another embodiment, the  
30 invention provides a conjugate containing CREKA (SEQ ID

NO: 1) or another tumor homing molecule that selectively homes to tumor vasculature, where the homing molecule selectively binds collagen IV such as a non-helical region of collagen IV or denatured collagen IV. In a further embodiment, the invention provides a conjugate containing CREKA (SEQ ID NO: 1) or another tumor homing molecule that selectively homes to tumor vasculature, where the homing molecule selectively binds non-helical collagen or a non-helical region of collagen in preference to the corresponding collagen in its native helical form. In still a further embodiment, the invention provides a conjugate containing CREKA (SEQ ID NO: 1) or another tumor homing molecule that selectively homes to tumor vasculature, where the homing molecule selectively binds denatured collagen IV in preference to native, helical collagen IV. Where preferential binding is specified, the tumor homing molecule generally exhibits at least two-fold or more increased binding to the non-helical form of collagen as compared to the corresponding native collagen.

Similar to the enhanced binding to denatured collagen exhibited by the CREKA peptide SEQ ID NO: 1, a "cryptic " monoclonal antibody site can be exposed in collagen IV by proteolysis; this site is recognized by monoclonal antibody HU1V26 and has been associated with angiogenic blood vessels yet not with quiescent vessels (Xu et al., J. Cell Biol. 154:1069-1079 (2001)). In one embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to tumor vasculature and selectively binds collagen and which is not monoclonal antibody HU1V26 or an antigen-binding

fragment thereof. In another embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to tumor vasculature and selectively binds collagen and which is not fibronectin or a fragment thereof, or an antibody or a fragment thereof. In a further embodiment, the invention provides a conjugate containing a homing molecule that selectively homes to tumor vasculature and selectively binds collagen and which is not fibronectin or a fragment thereof; an antibody or a fragment thereof; a collagenase or a fragment thereof, or a seminal fluid protein or a fragment thereof.

A variety of homing molecules that selectively home to tumor vasculature and selectively bind collagen such as non-helical collagen or collagen IV are useful in the conjugates of the invention. Such homing molecules include, without limitation, homing peptides and peptidomimetics. In one embodiment, the homing peptide or peptidomimetic portion of the conjugate has a length of at most 200 residues. In another embodiment, the homing peptide or peptidomimetic portion of the conjugate has a length of at most 50 residues. In a further embodiment, a conjugate of the invention contains a homing peptide or peptidomimetic that includes the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. In another embodiment, a conjugate of the invention contains a homing peptide or peptidomimetic that includes the amino acid sequence CREKA (SEQ ID NO: 1) or a peptidomimetic thereof. In still a further embodiment, a conjugate of

the invention contains a homing peptide that includes the amino acid sequence CREKA (SEQ ID NO: 1).

A variety of therapeutic agents are useful in the conjugates of the invention including, without  
5 limitation, cancer chemotherapeutic agents, cytotoxic agents, anti-angiogenic agents, polypeptides, nucleic acid molecules and small molecules. As non-limiting examples, the invention provides a conjugate containing a therapeutic agent linked to a homing molecule that  
10 selectively homes to tumor vasculature and selectively binds collagen, where the therapeutic agent is a cancer chemotherapeutic agent, cytotoxic agent, anti-angiogenic agent, polypeptide, nucleic acid molecule or small molecule. As further non-limiting examples, the  
15 invention provides a conjugate containing a therapeutic agent linked to a homing peptide or peptidomimetic that selectively homes to tumor vasculature and selectively binds collagen, where the therapeutic agent is a cancer chemotherapeutic agent, cytotoxic agent, anti-angiogenic  
20 agent, polypeptide, nucleic acid molecule or small molecule. As additional non-limiting examples, the invention provides a conjugate that contains a therapeutic agent linked to a homing peptide or peptidomimetic containing the amino acid sequence CREKA  
25 (SEQ ID NO: 1), or a conservative variant or peptidomimetic thereof, that selectively homes to tumor vasculature and selectively binds collagen, where the therapeutic agent is a cancer chemotherapeutic agent, cytotoxic agent, anti-angiogenic agent, polypeptide,  
30 nucleic acid molecule or small molecule. Also provided herein is a conjugate that contains a therapeutic agent

linked to a homing peptide or peptidomimetic containing the amino acid sequence CREKA (SEQ ID NO: 1), or a peptidomimetic thereof, that selectively homes to tumor vasculature and that selectively binds collagen, where  
5 the therapeutic agent is a cancer chemotherapeutic agent, cytotoxic agent, anti-angiogenic agent, polypeptide, nucleic acid molecule or small molecule. Further provided herein is a conjugate containing a therapeutic agent linked to a homing peptide containing the amino  
10 acid sequence CREKA (SEQ ID NO: 1) that selectively homes to tumor vasculature and selectively binds collagen, where the therapeutic agent is a cancer chemotherapeutic agent, cytotoxic agent, anti-angiogenic agent, polypeptide, nucleic acid molecule or small molecule.  
15 Any of these or other conjugates of the invention can optionally include a phage or other viral moiety.

The present invention further provides a method of directing a moiety to tumor vasculature in a subject  
20 by administering to the subject a conjugate which contains the moiety linked to a homing molecule that selectively homes to tumor vasculature and selectively binds collagen, thereby directing the moiety to tumor vasculature. In one embodiment, a method of the  
25 invention is practiced with a homing molecule that selectively homes to tumor vasculature and selectively binds non-helical collagen. In another embodiment, a method of the invention is practiced with a homing molecule that selectively homes to breast tumor  
30 vasculature and selectively binds collagen such as non-helical collagen. In yet another embodiment, a method of the invention is practiced with a homing

molecule that selectively homes to tumor vasculature and selectively binds collagen IV. In still another embodiment, a method of the invention is practiced with a homing molecule that selectively homes to tumor vasculature and selectively binds denatured collagen IV in preference to native collagen IV. In a further embodiment, a method of the invention is practiced with a homing molecule that selectively binds the alpha 2 chain of collagen IV. In yet a further embodiment, a method of the invention is practiced with a homing molecule and selectively binds collagen and selectively antigen-binding fragment thereof.

A variety of homing molecules can be useful in the methods of the invention for directing a moiety to tumor vasculature. Useful homing molecules include, yet are not limited to, homing peptides or peptidomimetics such as those including the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. In one embodiment, a method of the invention for directing a moiety to tumor vasculature is practiced with a homing molecule that includes the amino acid sequence CREKA (SEQ ID NO: 1), or a peptidomimetic thereof. In a further embodiment, a method of the invention for directing a moiety to tumor vasculature is practiced with a homing molecule that includes the amino acid sequence CREKA (SEQ ID NO: 1). A variety of moieties can be targeted to tumor vasculature according to a method of the invention including, without limitation, therapeutic agents, detectable agents and



phage. As non-limiting examples, therapeutic agents to be directed to tumor vasculature include cancer chemotherapeutic agents, cytotoxic agents, anti-angiogenic agents, polypeptides, nucleic acid molecules and small molecules.

The present invention additionally provides a method of reducing the number of tumor vessels in a subject by administering to the subject a conjugate which includes a therapeutic agent linked to a homing molecule that selectively homes to tumor vasculature and selectively binds collagen, thereby reducing the number of tumor vessels in the subject. A method of the invention can be useful, for example, for reducing the number of breast tumor vessels. In one embodiment, a method of the invention for reducing the number of tumor vessels is practiced with a homing molecule that selectively homes to tumor vasculature and selectively binds non-helical collagen. In another embodiment, a method of the invention for reducing the number of tumor vessels is practiced with a homing molecule that selectively homes to tumor vasculature and selectively binds collagen and which is not an antibody or antigen-binding fragment thereof. Homing molecules useful in the invention encompass, without limitation, homing peptides and peptidomimetics such as those including the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. A method of the invention can be practiced, for example, with a homing peptide or peptidomimetic containing the amino acid sequence CREKA (SEQ ID NO: 1) or a peptidomimetic thereof. A method of the invention also

can be practiced, for example, with a homing peptide containing the amino acid sequence CREKA (SEQ ID NO: 1). In a method of the invention for reducing the number of tumor vessels, a variety of therapeutic agents can be  
5 incorporated into the conjugate administered to the subject, including, for example, cancer chemotherapeutic agents, cytotoxic agents, and anti-angiogenic agents.

Also provided herein is a method of treating  
10 cancer in a subject by administering to the subject a conjugate which contains a therapeutic agent linked to a homing molecule that selectively homes to tumor vasculature and selectively binds collagen such as non-helical collagen or collagen IV. As a non-limiting  
15 example, a method of the invention can be useful for treating breast cancer. Homing molecules useful in the invention include those which selectively home to tumor vasculature and selectively bind collagen IV. Homing molecules useful in the invention further include those  
20 which selectively home to tumor vasculature and selectively bind the alpha 2 chain of collagen IV. In one embodiment, a method of the invention for treating cancer relies on a homing molecule that selectively homes to tumor vasculature and selectively binds collagen and  
25 which is not an antibody or antigen-binding fragment thereof.

A variety of homing molecules can be included in a conjugate useful for treating cancer according to a method of the invention. In one embodiment, a method of  
30 the invention is practiced with a conjugate that contains a homing peptide or peptidomimetic. In another

embodiment, a method of the invention is practiced with a conjugate in which the peptide or peptidomimetic portion of the conjugate has a length of at most 200 residues. In another embodiment, a method of the invention is practiced with a conjugate in which the peptide or peptidomimetic portion of the conjugate has a length of at most 50 residues. In yet another embodiment, a method of the invention is practiced with a conjugate which contains a homing peptide or peptidomimetic that includes the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. In still another embodiment, a method of the invention is practiced with a conjugate containing a homing peptide or peptidomimetic that includes the amino acid sequence CREKA (SEQ ID NO: 1), or a peptidomimetic thereof. In a further embodiment, a method of the invention is practiced with a conjugate containing a homing peptide which includes the amino acid sequence CREKA (SEQ ID NO: 1). A conjugate useful for treating cancer according to a method of the invention incorporates a therapeutic agent such as, without limitation, a cancer chemotherapeutic agent, cytotoxic agent, anti-angiogenic agent, polypeptide, nucleic acid molecule or small molecule and can optionally include one or more additional components such as a phage or other viral moiety.

The methods of the invention can be useful for treating a variety of cancers including, but not limited to, breast cancer, ovarian cancer, brain cancer such as glioblastoma or neuroblastoma, colon cancer, renal cancer, lung cancer, bladder cancer, prostate cancer and

melanoma. Cancers can be treated at early or late stage and at a pre- or post-metastatic stage. It is understood that the conjugates of the invention can be part of combination therapy, where the conjugates are  
5 simultaneously or sequentially administered with one or more other anti-cancer therapeutics.

Exemplified herein is a homing molecule which selectively homes to the matrix of breast tumor vasculature but which does not detectably home to  
10 non-tumor vasculature such as brain, heart, kidney, lung, pancreatic and breast vasculature. Additional homing molecules that, like CREKA (SEQ ID NO: 1) selectively home to tumor vasculature such as breast tumor vasculature can be identified using *in vivo* panning as  
15 described in U.S. Patent No. 5,622,699 coupled, if desired, with *ex vivo* selection or can be identified through *in vitro* assays such as the ability to selectively bind collagen as disclosed herein in Example III.

20 As used herein, the term "molecule" is used broadly to mean a polymeric or non-polymeric organic chemical such as a small molecule drug; a nucleic acid molecule such as an RNA, a DNA such as a cDNA or oligonucleotide; a peptide or peptidomimetic; or a  
25 protein such as a growth factor receptor or an antibody or fragment thereof such as an Fv, Fd, or Fab fragment or another antibody fragment containing the antigen-binding domain.

The term "homing molecule" as used herein, means any molecule that selectively homes *in vivo* to the vasculature of one or more tumors in preference to normal vasculature. Similarly, the term "homing peptide" or  
5 "homing peptidomimetic" means a peptide or peptidomimetic that selectively homes *in vivo* to the vasculature of one or more tumors in preference to normal vasculature. It is understood that a homing molecule that selectively homes *in vivo* to tumor vasculature can home to the  
10 vasculature of all tumors or can exhibit preferential homing to the vasculature of one or a subset of tumor types.

By "selectively homes" is meant that, *in vivo*, the homing molecule binds preferentially to tumor  
15 vasculature, such as breast tumor vasculature, as compared to non-tumoral vasculature. Such a homing molecule can selectively home, for example, to the matrix of tumor vasculature. Selective homing generally is characterized by at least a two-fold greater localization  
20 within tumor vasculature, such as breast tumor vasculature, as compared to several tissue types of non-tumor vasculature. A homing molecule can be characterized by 5-fold, 10-fold, 20-fold or more preferential localization to tumor vasculature as  
25 compared to several or many tissue types of non-tumoral vasculature, or as compared to most or all non-tumoral vasculature. Thus, it is understood that, in some cases, a homing molecule homes, in part, to the vasculature of one or more normal organs in addition to homing to breast  
30 and other tumor vasculature.

In one embodiment, a conjugate of the invention includes a homing molecule that selectively homes to tumor vasculature and selectively binds collagen and which is not an antibody or antigen-binding fragment  
5 thereof. The term "antibody" is an art-recognized term that refers to a peptide or polypeptide containing one or more complementarity determining regions (CDRs). See, for example, Borrahaeck, Antibody Engineering 2nd Edition, Oxford University Press, New York (1995).

10 In another embodiment, the peptide or peptidomimetic portion of the conjugate has a defined length. The peptide or peptidomimetic portion of the conjugate can have, for example, a length of at most 10, 20, 30, 40, 50, 100, 150, 200, 250, 300, 400,  
15 500, 600, 700, 800, 900, 1000 or 2000 residues. It is understood that the term "peptide or peptidomimetic portion of the conjugate" means total number of residues in the homing peptide or peptidomimetic and any contiguous protein component of the conjugate, such as a  
20 fused therapeutic protein or pro-apoptotic peptide.

The present invention also provides a conjugate containing a therapeutic agent and at least two homing molecules that each selectively homes to tumor vasculature and selectively binds collagen such as  
25 non-helical collagen or collagen IV. The two homing molecules can each independently contain, for example, the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. Further provided by the invention is a conjugate containing a  
30 therapeutic agent and at least ten homing molecules that

each selectively homes to tumor vasculature and selectively binds collagen such as non-helical collagen or collagen IV. The ten homing molecules can each independently contain, for example, the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. The invention also provides a conjugate containing a therapeutic agent and at least 100 homing molecules that each selectively homes to tumor vasculature and selectively binds collagen such as non-helical collagen or collagen IV. The 100 homing molecules included in the conjugate can each independently contain, for example, the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. Any of the above conjugates of the invention containing multiple homing molecules can optionally include a phage or other viral moiety.

A conjugate of the invention containing multiple homing molecules can include, for example, two or more, three or more, five or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, 100 or more, 200 or more, 300 or more, 400 or more, 500 or more, or 1000 or more homing molecules. In one embodiment, the conjugate includes homing molecules that all have an identical amino acid sequence. In another embodiment, the conjugate includes homing molecules having two or more non-identical amino acid sequences. Moieties useful in a conjugate of the invention incorporating multiple homing molecules include, without limitation, phage, retroviruses, adenoviruses, adeno-associated viruses and other viruses, cells, liposomes, polymeric matrices, non-polymeric matrices, particles

such as gold particles, microdevices, nanodevices, and nano-scale semiconductor materials.

A conjugate of the invention can contain, for example, a liposome or other polymeric matrix linked to at least two homing molecules which each selectively homes to tumor vasculature and selectively binds collagen. If desired, the liposome or other polymeric matrix can be linked to at least ten, at least 100 or at least 1000 homing molecules which each selectively homes to tumor vasculature and selectively binds collagen. Homing molecules useful in such a conjugate can independently include, for example, the amino acid sequence CREKA (SEQ ID NO: 1), or a conservative variant or peptidomimetic of this sequence. Liposomes can be useful in such conjugates; liposomes consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer (Gregoriadis, Liposome Technology, Vol. 1 (CRC Press, Boca Raton, FL (1984))). The liposome or other polymeric matrix can optionally include another component such as, without limitation, a therapeutic agent, cancer chemotherapeutic agent, cytotoxic agent, anti-angiogenic agent, polypeptide or nucleic acid molecule.

The present invention provides methods of directing a moiety to tumor vasculature. As used herein, the term "moiety" is used broadly to mean a physical, chemical, or biological material that generally imparts a biologically useful function to a linked molecule. A moiety can be any natural or nonnatural material



including, without limitation, a biological material, such as a cell, phage or other virus; an organic chemical such as a small molecule; a radionuclide; a nucleic acid molecule or oligonucleotide; a polypeptide; or a peptide  
5 or peptidomimetic. Moieties useful in the invention include, yet are not limited to, therapeutic agents such as cancer chemotherapeutic agents, cytotoxic agents, pro-apoptotic agents, and anti-angiogenic agents; detectable labels and imaging agents; and tags or other  
10 insoluble supports. Moieties useful in the invention further include, without limitation, phage and other viruses, cells, liposomes, polymeric matrices, non-polymeric matrices or particles such as gold particles, microdevices and nanodevices, and nano-scale  
15 semiconductor materials. These and other moieties known in the art can be components of a conjugate of the invention.

In one embodiment, the moiety incorporated into  
20 a conjugate of the invention is a therapeutic agent. As used herein, the term "therapeutic agent" means a molecule which has one or more biological activities in a normal or pathologic tissue. A variety of therapeutic agents can be included in a conjugate of the invention.  
25 In one embodiment, a conjugate of the invention contains a cancer chemotherapeutic agent. As used herein, a "cancer chemotherapeutic agent" is a chemical agent that inhibits the proliferation, growth, life-span or metastatic activity of cancer cells. Such a cancer  
30 chemotherapeutic agent can be, without limitation, a taxane such as docetaxel; an anthracyclin such as doxorubicin; an alkylating agent; a vinca alkaloid; an

anti-metabolite; a platinum agent such as cisplatin or carboplatin; a steroid such as methotrexate; an antibiotic such as adriamycin; a isofamide; or a selective estrogen receptor modulator; an antibody such  
5 as trastuzumab.

Taxanes are chemotherapeutic agents useful in the conjugates of the invention. Useful taxanes include, without limitation, docetaxel (Taxotere; Aventis Pharmaceuticals, Inc.; Parsippany, NJ) and paclitaxel  
10 (Taxol; Bristol-Myers Squibb; Princeton, NJ). See, for example, Chan et al., J. Clin. Oncol. 17:2341-2354 (1999), and Paridaens et al., J. Clin. Oncol. 18:724 (2000).

A cancer chemotherapeutic agent useful in a  
15 conjugate of the invention also can be an anthracyclin such as doxorubicin, idarubicin or daunorubicin. Doxorubicin is a commonly used cancer chemotherapeutic agent and can be useful, for example, for treating breast cancer (Stewart and Ratain, In: "Cancer: Principles and  
20 practice of oncology" 5th ed., chap. 19 (eds. DeVita, Jr., et al.; J.P. Lippincott 1997); Harris et al., In "Cancer: Principles and practice of oncology," *supra*, 1997). In addition, doxorubicin has anti-angiogenic activity (Folkman, Nature Biotechnology 15:510 (1997);  
25 Steiner, In "Angiogenesis: Key principles-Science, technology and medicine," pp. 449-454 (eds. Steiner et al.; Birkhauser Verlag, 1992)), which can contribute to its effectiveness in treating cancer.

An alkylating agent such as melphalan or chlorambucil also can be a cancer chemotherapeutic agent useful in a conjugate of the invention. Similarly, a vinca alkaloid such as vindesine, vinblastine or  
5 vinorelbine; or an antimetabolite such as 5-fluorouracil, 5-fluorouridine or a derivative thereof can be a cancer chemotherapeutic agent useful in a conjugate of the invention.

A platinum agent also can be a cancer  
10 chemotherapeutic agent useful in the conjugates of the invention. Such a platinum agent can be, for example, cisplatin or carboplatin as described, for example, in Crown, Seminars in Oncol. 28:28-37 (2001). Other cancer chemotherapeutic agents useful in a conjugate of the  
15 invention include, without limitation, methotrexate, mitomycin-C, adriamycin, ifosfamide and ansamycins.

A cancer chemotherapeutic agent for treatment of breast cancer and other hormonally-dependent cancers also can be an agent that antagonizes the effect of  
20 estrogen, such as a selective estrogen receptor modulator or an anti-estrogen. The selective estrogen receptor modulator, tamoxifen, is a cancer chemotherapeutic agent that can be used in a conjugate of the invention for treatment of breast cancer (Fisher et al., J. Natl.  
25 Cancer Instit. 90:1371-1388 (1998)).

A therapeutic agent useful in a conjugate of the invention can be an antibody such as a humanized monoclonal antibody. As an example, the anti-epidermal growth factor receptor 2 (HER2) antibody, trastuzumab

(Herceptin; Genentech, South San Francisco, CA) is a therapeutic agent useful in a conjugate of the invention for treating HER2/neu overexpressing breast cancers (White et al., Annu. Rev. Med. 52:125-141 (2001)).

5           A therapeutic agent useful in the invention also can be a cytotoxic agent, which, as used herein, is any molecule that directly or indirectly promotes cell death. Cytotoxic agents useful in the invention include, without limitation, small molecules, polypeptides,  
10 peptides, peptidomimetics, nucleic acid molecules, cells and viruses. As non-limiting examples, cytotoxic agents useful in the invention include cytotoxic small molecules such as doxorubicin, docetaxel or trastuzumab; antimicrobial peptides such as those described further  
15 below; pro-apoptotic polypeptides such as caspases and toxins, for example, caspase-8; diphtheria toxin A chain, *Pseudomonas* exotoxin A, cholera toxin, ligand fusion toxins such as DAB389EGF, ricinus communis toxin (ricin); and cytotoxic cells such as cytotoxic T cells. See, for  
20 example, Martin et al., Cancer Res. 60:3218-3224 (2000); Kreitman and Pastan, Blood 90:252-259 (1997); Allam et al., Cancer Res. 57:2615-2618 (1997); and Osborne and Coronado-Heinsohn, Cancer J. Sci. Am. 2:175 (1996). One skilled in the art understands that these and additional  
25 cytotoxic agents described herein or known in the art can be useful in the conjugates and methods of the invention.

          In one embodiment, a therapeutic agent is a therapeutic polypeptide. As used herein, a therapeutic polypeptide is any polypeptide with a biologically useful  
30 function. Therapeutic polypeptides useful in the

invention encompass, without limitation, cytokines, antibodies, cytotoxic polypeptides; pro-apoptotic polypeptides; and anti-angiogenic polypeptides. As non-limiting examples, a therapeutic polypeptide useful  
5 in the invention can be a cytokine such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), tumor necrosis factor- $\beta$  (TNF- $\beta$ ), granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interferon  $\alpha$  (IFN- $\alpha$ ); interferon  $\gamma$  (IFN- $\gamma$ ), interleukin-1  
10 (IL-1), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-10 (IL-10), interleukin-12 (IL-12), lymphotactin (LTN) or dendritic cell chemokine 1 (DC-CK1); an anti-HER2 antibody or fragment thereof; a  
15 cytotoxic polypeptide including a toxin or caspase, for example, diphtheria toxin A chain, *Pseudomonas* exotoxin A, cholera toxin, a ligand fusion toxin such as DAB389EGF or ricin; or an anti-angiogenic polypeptide such as angiostatin, endostatin, thrombospondin, platelet  
20 factor 4; anastellin; or one of those described further herein or known in the art (see below). It is understood that these and other polypeptides with biological activity can be a "therapeutic polypeptide" useful in the invention.

25 A therapeutic agent useful in a conjugate of the invention also can be an anti-angiogenic agent. As used herein, the term "anti-angiogenic agent" means a molecule that reduces or prevents angiogenesis, which is the growth and development of blood vessels. A variety  
30 of anti-angiogenic agents are useful in the invention and can be prepared by routine methods. Such anti-angiogenic

agents include, without limitation, small molecules; proteins such as dominant negative forms of angiogenic factors, transcription factors and antibodies; peptides and peptidomimetics; and nucleic acid molecules including  
 5 ribozymes, antisense oligonucleotides, and nucleic acid molecules encoding, for example, dominant negative forms of angiogenic factors and receptors, transcription factors, and antibodies and antigen-binding fragments thereof. See, for example, Hagedorn and Bikfalvi, Crit.  
 10 Rev. Oncol. Hematol. 34:89-110 (2000), and Kirsch et al., J. Neurooncol. 50:149-163 (2000).

Vascular endothelial growth factor (VEGF) has been shown to be important for angiogenesis in many types of cancer, including breast cancer angiogenesis *in vivo*  
 15 (Borgstrom et al., Anticancer Res. 19:4213-4214 (1999)). The biological effects of VEGF include stimulation of endothelial cell proliferation, survival, migration and tube formation, and regulation of vascular permeability. An anti-angiogenic agent useful in the invention can be,  
 20 for example, an inhibitor or neutralizing antibody that reduces the expression or signaling of VEGF or another angiogenic factor, for example, an anti-VEGF neutralizing monoclonal antibody (Borgstrom et al., *supra*, 1999). An anti-angiogenic agent also can inhibit another angiogenic  
 25 factor such as a member of the fibroblast growth factor family such as FGF-1 (acidic), FGF-2 (basic), FGF-4 or FGF-5 (Slavin et al., Cell Biol. Int. 19:431-444 (1995); Folkman and Shing, J. Biol. Chem. 267:10931-10934 (1992)) or an angiogenic factor such as angiopoietin-1, a factor  
 30 that signals through the endothelial cell-specific Tie2 receptor tyrosine kinase (Davis et al., Cell 87:1161-1169

(1996); and Suri et al., Cell 87:1171-1180 (1996)), or the receptor of one of these angiogenic factors. It is understood that a variety of mechanisms can act to inhibit activity of an angiogenic factor including,  
 5 without limitation, direct inhibition of receptor binding, indirect inhibition by reducing secretion of the angiogenic factor into the extracellular space, or inhibition of expression, function or signaling of the angiogenic factor.

10           A variety of other molecules also can function as anti-angiogenic agents useful in the invention including, without limitation, angiostatin; a kringle peptide of angiostatin; endostatin; anastellin, heparin-binding fragments of fibronectin; modified forms  
 15 of antithrombin; collagenase inhibitors; basement membrane turnover inhibitors; angiostatic steroids; platelet factor 4 and fragments and peptides thereof; thrombospondin and fragments and peptides thereof; and doxorubicin (O'Reilly et al., Cell 79:315-328 (1994));  
 20 O'Reilly et al., Cell 88:277-285 (1997); Homandberg et al., Am. J. Path. 120:327-332 (1985); Homandberg et al., Biochim. Biophys. Acta 874:61-71 (1986); and O'Reilly et al., Science 285:1926-1928 (1999)). Commercially available anti-angiogenic agents useful in the invention  
 25 include, for example, angiostatin, endostatin, metastatin and 2ME2 (EntreMed; Rockville, MD); anti-VEGF antibodies such as Avastin (Genentech; South San Francisco, CA); and VEGFR-2 inhibitors such as SU5416, a small molecule inhibitor of VEGFR-2 (SUGEN; South San Francisco, CA) and  
 30 SU6668 (SUGEN), a small molecule inhibitor of VEGFR-2, platelet derived growth factor and fibroblast growth

factor I receptor. It is understood that these and other anti-angiogenic agents can be prepared by routine methods and are encompassed by the term "anti-angiogenic agent" as used herein.

5

A therapeutic agent useful in the invention also can be an antimicrobial peptide. Thus, the invention further provides a conjugate in which a homing molecule that selectively homes to tumor vasculature and  
10 selectively binds collagen is linked to an antimicrobial peptide, where the conjugate is selectively internalized by tumor vasculature and exhibits a high toxicity to the tumor vasculature, and where the antimicrobial peptide has low mammalian cell toxicity when not linked to the  
15 homing molecule. As used herein, the term "antimicrobial peptide" means a naturally occurring or synthetic peptide having antimicrobial activity, which is the ability to kill or slow the growth of one or more microbes and which has low mammalian cell toxicity when not linked to a  
20 homing molecule. An antimicrobial peptide can, for example, kill or slow the growth of one or more strains of bacteria including a Gram-positive or Gram-negative bacteria, or a fungi or protozoa. Thus, an antimicrobial peptide can have, for example, bacteriostatic or  
25 bacteriocidal activity against, for example, one or more strains of *Escherichia coli*, *Pseudomonas aeruginosa* or *Staphylococcus aureus*. While not wishing to be bound by the following, an antimicrobial peptide can have biological activity due to the ability to form ion  
30 channels through membrane bilayers as a consequence of self-aggregation.



An antimicrobial peptide is typically highly basic and can have a linear or cyclic structure. As discussed further below, an antimicrobial peptide can have an amphipathic  $\alpha$ -helical structure (see U.S. Patent 5 5,789,542; Javadpour et al., J. Med. Chem. 39:3107-3113 (1996); and Blondelle and Houghten, Biochem. 31: 12688-12694 (1992)). An antimicrobial peptide also can be, for example, a  $\beta$ -strand/sheet-forming peptide as described in Mancheno et al., J. Peptide Res. 51:142-148 10 (1998).

An antimicrobial peptide can be a naturally occurring or synthetic peptide. Naturally occurring antimicrobial peptides have been isolated from biological sources such as bacteria, insects, amphibians, and 15 mammals and are thought to represent inducible defense proteins that can protect the host organism from bacterial infection. Naturally occurring antimicrobial peptides include the gramicidins, magainins, mellitins, defensins and cecropins (see, for example, Maloy and 20 Kari, Biopolymers 37:105-122 (1995); Alvarez-Bravo et al., Biochem. J. 302:535-538 (1994); Bessalle et al., FEBS 274:151-155 (1990); and Blondelle and Houghten in Bristol (Ed.), Annual Reports in Medicinal Chemistry pages 159-168 Academic Press, San Diego). An 25 antimicrobial peptide also can be an analog of a natural peptide, especially one that retains or enhances amphipathicity (see below).

An antimicrobial peptide incorporated into a conjugate of the invention has low mammalian cell 30 toxicity when not linked to a tumor homing molecule.

Mammalian cell toxicity readily can be assessed using routine assays. As an example, mammalian cell toxicity can be assayed by lysis of human erythrocytes *in vitro* as described in Javadpour et al., *supra*, 1996. An antimicrobial peptide having low mammalian cell toxicity is not lytic to human erythrocytes or requires concentrations of greater than 100  $\mu\text{M}$  for lytic activity, preferably concentrations greater than 200, 300, 500 or 1000  $\mu\text{M}$ .

10 In one embodiment, the invention provides a conjugate in which the antimicrobial peptide portion promotes disruption of mitochondrial membranes when internalized by eukaryotic cells. In particular, such an antimicrobial peptide preferentially disrupts

15 mitochondrial membranes as compared to eukaryotic membranes. Mitochondrial membranes, like bacterial membranes but in contrast to eukaryotic plasma membranes, have a high content of negatively charged phospholipids. An antimicrobial peptide can be assayed for activity in

20 disrupting mitochondrial membranes using, for example, an assay for mitochondrial swelling or another assay well known in the art.  $\text{D}(\text{KLAKLAK})_2$ , for example, is an antimicrobial peptide which induces marked mitochondrial swelling at a concentration of 10  $\mu\text{M}$ , significantly less

25 than the concentration required to kill eukaryotic cells. An antimicrobial peptide that induces significant mitochondrial swelling at, for example, 50  $\mu\text{M}$ , 40  $\mu\text{M}$ , 30  $\mu\text{M}$ , 20  $\mu\text{M}$ , 10  $\mu\text{M}$ , or less, is considered a peptide that promotes disruption of mitochondrial membranes.

An antimicrobial peptide can include, for example, the sequence (KLAKLAK)<sub>2</sub> (SEQ ID NO: 20), (KLAKKLA)<sub>2</sub> (SEQ ID NO: 21), (KAAKKAA)<sub>2</sub> (SEQ ID NO: 22), or (KLGKKLG)<sub>3</sub> (SEQ ID NO: 23), and, in one embodiment, 5 includes the sequence <sub>D</sub>(KLAKLAK)<sub>2</sub>. A conjugate of the invention, which contains a homing molecule that selectively homes to tumor vasculature and selectively binds collagen linked to an antimicrobial peptide, can have, for example, the sequence CREKA-GG-<sub>D</sub>(KLAKLAK)<sub>2</sub>.

10

Antimicrobial peptides generally have random coil conformations in dilute aqueous solutions, yet high levels of helicity can be induced by helix-promoting solvents and amphipathic media such as micelles, 15 synthetic bilayers or cell membranes.  $\alpha$ -Helical structures are well known in the art, with an ideal  $\alpha$ -helix characterized by having 3.6 residues per turn and a translation of 1.5 Å per residue (5.4Å per turn; see Creighton, Proteins: Structures and Molecular Properties 20 W.H Freeman, New York (1984)). In an amphipathic  $\alpha$ -helical structure, polar and non-polar amino acid residues are aligned into an amphipathic helix, which is an  $\alpha$ -helix in which the hydrophobic amino acid residues are predominantly on one face, with hydrophilic residues 25 predominantly on the opposite face when the peptide is viewed along the helical axis.

Antimicrobial peptides of widely varying sequence have been isolated, sharing an amphipathic  $\alpha$ -helical structure as a common feature (Saberwal et al., 30 Biochim. Biophys. Acta 1197:109-131 (1994)). Analogs of native peptides with amino acid substitutions predicted

to enhance amphipathicity and helicity typically have increased antimicrobial activity. In general, analogs with increased antimicrobial activity also have increased cytotoxicity against mammalian cells (Maloy et al.,  
5 Biopolymers 37:105-122 (1995)).

As used herein in reference to an antimicrobial peptide, the term "amphipathic  $\alpha$ -helical structure" means an  $\alpha$ -helix with a hydrophilic face containing several polar residues at physiological pH and a hydrophobic face  
10 containing nonpolar residues. A polar residue can be, for example, a lysine or arginine residue, while a nonpolar residue can be, for example, a leucine or alanine residue. An antimicrobial peptide having an amphipathic  $\alpha$ -helical structure generally has an  
15 equivalent number of polar and nonpolar residues within the amphipathic domain and a sufficient number of basic residues to give the peptide an overall positive charge at neutral pH (Saberwal et al., Biochim. Biophys. Acta  
1197:109-131 (1994)). One skilled in the art understands  
20 that helix-promoting amino acids such as leucine and alanine can be advantageously included in an antimicrobial peptide of the invention (see, for example, Creighton, *supra*, 1984). Synthetic, antimicrobial peptides having an amphipathic  $\alpha$ -helical structure are  
25 known in the art, for example, as described in U.S. Patent No. 5,789,542 to McLaughlin and Becker.

It is understood by one skilled in the art of medicinal oncology that these and other agents are useful therapeutic agents, which can be used separately or  
30 together in the conjugates and methods of the invention.

Thus, it is understood that a conjugate of the invention can contain one or more of such therapeutic agents and that additional components can be included as part of the conjugate, if desired. As a non-limiting example, it can  
5 be desirable in some cases to utilize an oligopeptide spacer between the homing molecule and the therapeutic agent (Fitzpatrick and Garnett, Anticancer Drug Des. 10:1-9 (1995)).

A variety of routes of administration are  
10 useful in the methods of the invention. Such routes include both systemic and local administration and encompass, without limitation, oral administration, intravenous injection, intraperitoneal injection, intramuscular injection, subcutaneous injection,  
15 transdermal diffusion or electrophoresis, local injection; extended release delivery devices such as locally implanted extended release devices and bioerodible or reservoir-based implants.

Also provided herein is a method of imaging  
20 tumor vasculature in a subject by (a) administering to the subject a conjugate containing a detectable agent linked to a homing molecule that selectively homes to tumor vasculature and selectively binds collagen; and (b) detecting the conjugate, thereby imaging tumor  
25 vasculature. In one embodiment, an imaging method of the invention is practiced with a homing molecule that selectively homes to tumor vasculature and selectively binds non-helical collagen. In another embodiment, an imaging method of the invention is practiced with a  
30 homing molecule that selectively homes to tumor

vasculature and selectively binds collagen IV. In yet a further embodiment, an imaging method of the invention is practiced with a homing molecule that selectively homes to tumor vasculature and selectively binds denatured collagen IV in preference to native collagen IV. In another embodiment, an imaging method that selectively homes to tumor vasculature and selectively binds collagen and which is not an antibody or antigen-binding fragment thereof. In another embodiment, a method of the invention is used to image breast tumor vasculature.

A variety of homing molecules can be useful in the imaging methods of the invention, including homing peptides and peptidomimetics such as homing peptides or peptidomimetics containing the amino acid sequence CREKA (SEQ ID NO: 1) or a conservative variant or peptidomimetic thereof. In one embodiment, the invention provides an imaging method that relies on a homing peptide or peptidomimetic containing the amino acid sequence CREKA (SEQ ID NO: 1) or a peptidomimetic thereof. In another embodiment, the invention provides an imaging method that relies on a homing peptide containing the amino acid sequence CREKA (SEQ ID NO: 1). Detectable agents useful in the imaging methods of the invention encompass, yet are not limited to, fluorophores and radionuclides, including radionuclides such as indium-111, technetium-99, carbon-11 and carbon-13.

The methods of the invention for imaging tumor vasculature can be useful for detecting the presence of tumor vasculature associated with a variety of tumors,

including breast, ovarian, brain, colon, kidney, lung, bladder and prostate tumors and melanomas. Following administration of a conjugate of the invention containing a detectable agent, tumor vasculature is visualized. If  
5 the image is positive for the presence of tumor blood vessels, the tumor can be evaluated for size and quantity of vascular infiltration. These results provide valuable information to the clinician with regard to the stage of development of the cancer and the presence or probability  
10 of metastasis.

In a method of imaging tumor lymphatic vasculature, the conjugate administered contains a detectable agent that allows detection or visualization of vasculature in and around tumors, for example in and  
15 around breast tumors. For *in vivo* diagnostic imaging of tumor vasculature, a homing molecule is linked to a detectable agent that, upon administration to the subject, is detectable external to the subject. Such a detectable agent can be, for example, a gamma ray  
20 emitting radionuclide such as indium-113, indium-115 or technetium-99; following administration to a subject, the conjugate can be visualized using a solid scintillation detector.

A variety of detectable agents are useful in  
25 the methods of the invention. As used herein, the term "detectable agent" refers to any molecule which can be administered *in vivo* and subsequently detected. Detectable agents useful in the conjugates and imaging methods of the invention include yet are not limited to  
30 radiolabels and fluorescent molecules. Exemplary

radionuclides include indium-111, technetium-99,  
carbon-11, and carbon-13. Fluorescent molecules useful  
in the invention encompass, without limitation,  
fluorescein, allophycocyanin, phycoerythrin, rhodamine,  
5 and Texas red.

As disclosed herein, peptide SEQ ID NO: 1  
recognizes a receptor expressed in the matrix of tumor  
blood vessels and thereby selectively homes to the matrix  
10 of tumor vasculature. Furthermore, the matrix protein  
recognized by SEQ ID NO: 1 does not serve as a receptor  
for significant homing to the vasculature of a variety of  
normal tissues including brain, heart, kidney, lung and  
pancreas. The binding of SEQ ID NO: 1 to a target  
15 receptor expressed in the matrix of tumor vasculature  
forms the basis for the selective homing activity of  
peptide SEQ ID NO: 1 and related peptides,  
peptidomimetics and other molecules. Based on this  
discovery, it is clear that molecules structurally  
20 unrelated to SEQ ID NO: 1 but which also bind the same  
target receptor also have the same characteristic of  
selective homing to tumor vasculature. Such molecules  
can be identified by the ability to selectively bind to,  
or compete for binding to, collagen IV or another  
25 collagen or fragment bound by SEQ ID NO: 1. Thus, the  
invention provides conjugates containing a molecule that  
selectively binds collagen IV or another collagen or  
other receptor bound by peptide SEQ ID NO: 1; such a  
molecule also is characterized by the ability to  
30 selectively home to tumor vasculature. In one  
embodiment, the invention provides a conjugate containing



a homing peptide or peptidomimetic having a length of at most 4, 5, 6, 7, 8, 9, 10, 15, 20, 40, 60 or 100 residues, that specifically binds denatured collagen IV or another receptor bound by peptide SEQ ID NO: 1.

5           The present invention further provides a method of identifying a tumor homing molecule that selectively homes to tumor vasculature by contacting a substantially purified collagen, or fragment thereof, with one or more molecules; and determining specific binding of a molecule  
10 to the substantially purified collagen or fragment thereof, where the presence of specific binding identifies the molecule as a tumor homing molecule that selectively homes to tumor vasculature. A method of the invention can further include, if desired, the steps of  
15 administering the collagen binding molecule *in vivo*; and determining binding of the collagen binding molecule to tumor vasculature. If desired, the substantially purified collagen useful in the invention can be immobilized on a support.

20           Substantially purified collagens useful in the invention include, yet are not limited to, collagen type I, II, IV and X and further include, without limitation, any collagen alpha 2 chain. In one embodiment, a screening method of the invention is  
25 practiced with a substantially purified non-helical collagen, or fragment thereof. In another embodiment, a screening method of the invention is practiced with substantially purified collagen or fragment thereof which is denatured, for example, by boiling. In a further  
30 embodiment, a screening method of the invention is

practiced with substantially purified collagen type IV or a fragment thereof. The method can be practiced, for example, with denatured collagen type IV, or a fragment thereof, or with the alpha 2 chain of collagen type IV or  
5 a fragment thereof. As a non-limiting example, a method of the invention can be practiced with substantially purified collagen IV which is denatured by boiling.

The methods of the invention can be practiced with any species, type or isoform of collagen including  
10 those described herein above, and further can be practiced with any fragment of collagen which contains Gly-X-Y repeats or which can selectively bind CREKA (SEQ ID NO: 1). Thus, substantially purified collagen useful in the screening methods of the invention include,  
15 without limitation, mammalian collagens such as human, bovine, rat and mouse collagens; various types of collagen including collagen type I, type II, type IV and type X; and various collagen isoforms including a collagen containing one or more  $\alpha 2$  chains such as  $\alpha 2(\text{IV})$   
20 chains. Substantially purified collagens useful in the invention include those purified from normal tissue or tumor sources as well as recombinant polypeptides and can be prepared by routine methods.

Collagen fragments also can be useful in the  
25 screening methods of the invention. Such fragments encompass fragments that include Gly-X-Y repeats, for example, fragments of a collagen alpha 2 chain. In one embodiment, a collagen fragment useful in the invention is homologous to residues 338 to 476 of the human  
30 collagen IV alpha 2 chain shown in Figure 7. Such a

fragment can have, for example, greater than 40%, 50%, 60%, 70%, 80%, 90% or 95% identity with residues 338 to 476 of SEQ ID NO: 4. It is understood that these and other fragments that retain selective binding for the  
5 CREKA (SEQ ID NO: 1) peptide are useful in the screening methods of the invention.

The present invention further provides a method of identifying a tumor homing molecule that selectively homes to tumor vasculature by contacting a substantially  
10 purified polypeptide containing Gly-X-Y repeats, or a fragment thereof, with one or more molecules; and determining specific binding of a molecule to the substantially purified polypeptide or fragment thereof, where the presence of specific binding identifies the  
15 molecule as a tumor homing molecule that selectively homes to tumor vasculature. A method of the invention can further include, if desired, the steps of administering the polypeptide binding molecule *in vivo*; and determining binding of the molecule to tumor  
20 vasculature. In one embodiment, the substantially purified polypeptide or fragment thereof is homologous to SEQ ID NO: 2. In another embodiment, the substantially purified polypeptide or fragment thereof is denatured, for example, by boiling.

25

The following examples are intended to illustrate but not limit the present invention.

## EXAMPLE 1

PHAGE EXPRESSING THE PENTAPEPTIDE CREKA (SEQ ID NO: 1)  
HOME TO BREAST CANCER TISSUE

This example describes identification of the  
5 CREKA (SEQ ID NO: 1) tumor homing peptide.

To identify phage that selectively home to  
tumor vasculature, a phage-displayed peptide library was  
injected intravenously into two month-old MMTV-PyMT mice;  
10 bound phage were subsequently recovered from breast tumor  
tissue. The number of phage recovered from breast tissue  
increased about 100-fold after three rounds of injection  
and recovery; additional rounds of injection and recovery  
did not further improve the selectivity. Sequence  
15 analysis showed that phage displaying the peptide CREKA  
(SEQ ID NO: 1) were significantly enriched in the phage  
pool recovered after three rounds of screening,  
representing about 20% of phage present in the pool.

Phage preparations ( $1 \times 10^9$  plaque forming  
20 units (pfu)) of CREKA (SEQ ID NO: 1)-displaying phage  
were injected into tumor-bearing mice and subsequently  
recovered from breast tumors and a variety of normal  
tissues. As shown in Figure 1A, CREKA (SEQ ID NO: 1)  
phage homed to breast tissue about 130 times more  
25 efficiently than non-recombinant T7 phage. The CREKA  
(SEQ ID NO: 1) phage also homed to the vasculature of  
MDA-MB-435 human breast cancer cell xenografts grown in  
the mammary fat pad of nude mice with a 20-fold  
specificity over non-recombinant phage as shown in

Figure 1B. The CREKA (SEQ ID NO: 1) phage did not home to a variety of normal tissues, including the pancreas, brain, kidneys and heart of MMTV PyMT tumor-bearing mice (see Figure 1A). Furthermore, the CREKA (SEQ ID NO: 1)-displaying phage also did not home to the vasculature of small xenograft tumors growing in the mammary fat pad. Tumor homing of CREKA (SEQ ID NO: 1)-displaying phage was specific by the criterion of ligand inhibition; co-injection of synthetic free CREKA (SEQ ID NO: 1) peptide inhibited CREKA (SEQ ID NO: 1)-displaying phage recovery from breast cancer tissue while an excess of an unrelated control peptide did not inhibit homing of CREKA (SEQ ID NO: 1)-phage. Similarly, free CREKA (SEQ ID NO: 1) peptide also did not inhibit homing of phage displaying the control peptide.

A peptide library with the general structure of CX<sub>2</sub>C, where C is cysteine and X is any amino acid, was constructed in T7 phage essentially as follows. Briefly, complementary oligonucleotides with NNK codons encoding a random peptide insert were annealed. The resulting double stranded DNA had 5' EcoRI and 3' HindIII overhangs, which were phosphorylated with T4 polynucleotide kinase (Novagen; Madison, Wisconsin) and ligated into 1 µg of T7Select415-1b vector arms (Novagen). The ligate was directly added to 50 µl of packaging extract and incubated for two hours. The total number of recombinants obtained was ~10<sup>8</sup> as measured by formation of plaque forming units. Recombinants were amplified in 500 ml of liquid culture; purification of phage particles and sequencing of single stranded phage DNA was performed as described in Essler and Ruoslahti, Proc. Natl. Acad.

Sci., USA 99:2252-2257 (1999). The source of all materials not specified in this and further examples was Sigma (St. Louis, MO).

In vivo phage display screens were performed with 65 to 75 day old transgenic MMTV PyMT mice obtained from Dr. William Muller. MDA-MB-435 tumors were generated as described in Laakkonen et al., Nat. Med. 7:751-755 (2002). Briefly,  $10^6$  MDA-MB-435 cells in 100  $\mu$ l phosphate buffered saline (PBS) were injected into the mammary fat pad of nude mice. Tumors were grown for five weeks before the animals were used in *in vivo* studies. In vivo phage screening was performed as described in Porkka et al., Proc. Natl. Acad. Sci., USA 99:7444-7449 (2002), with a few modifications. Briefly, mice were anesthetized with avertin and then injected intravenously with  $10^9$  pfu of the CX<sub>7</sub>C library. Mice were perfused through the heart with 10 ml of PBS seven minutes after the injection. Tumor tissue was then excised, weighed, and a cell suspension was made using a Medimachine (DAKO; Denmark). The resulting single cells were centrifuged at 1500 rpm and washed five times with 5 ml PBS. Cell-adherent phage particles were recovered by infecting BL21 bacteria (Novagen), and the number of phage quantified by plaque assay.

Synthetic CREKA (SEQ ID NO: 1) peptide was synthesized in the peptide synthesis facility at The Burnham Institute using Fmoc chemistry on a solid-phase synthesizer. The peptide was purified by HPLC, and the sequence and structure confirmed by mass spectrometry.

These results demonstrate that the CREKA (SEQ ID NO: 1) peptide confers on an attached moiety such as a phage the ability to be selectively directed in vivo to tumor vasculature.

5

## EXAMPLE 2

TISSUE LOCALIZATION OF CREKA (SEQ ID NO: 1)-DISPLAYING  
PHAGE AND CREKA (SEQ ID NO: 1) PEPTIDE

This example describes the selective homing of CREKA (SEQ ID NO: 1)-displaying phage and labeled CREKA  
10 (SEQ ID NO: 1) peptide.

Immunoperoxidase staining revealed CREKA (SEQ ID NO: 1) phage in MMTV PyMT tumors (Figure 2A) and in MDA-MB-435 xenografts (Figure 2B) 15 minutes after intravenous injection. In contrast, similarly injected  
15 non-recombinant T7 phage could not be detected in the tumors (Figure 2C). Furthermore, the IgG control antibody staining was negative (Figure 2D), and no CREKA (SEQ ID NO: 1)-displaying phage were detected in control normal organs such as the brain, kidney and heart  
20 (Figures 2E, 2F and 2G, respectively). The liver stained positive for the CREKA (SEQ ID NO: 1) phage as shown in panel 2H and also stained positive for control phage, indicating non-specific uptake of the phage by the reticuloendothelial system, as has been observed  
25 previously (Pasqualini et al., Cancer Res. 60:722-727 (2000)).

Similarly, fluorescein (FITC) and rhodamine labeled CREKA (SEQ ID NO: 1) peptides also were detected

in MMTV PyMT tumors 15 minutes after intravenous injection but were not observed in normal tissues such as the brain or liver (compare Figure 3A with Figure 3B and 3C). The peptides were found primarily in the tumor periphery 15 minutes after the injection but could be detected in the entire tumor after two hours, localizing outside the blood vessels as shown in Figure 3D. As expected, control organs such as the heart, injected with rhodamine-labeled CREKA (SEQ ID NO: 1) and FITC-tomato-lectin, which stains blood vessels, were negative for CREKA (SEQ ID NO: 1) peptide staining (see Figure 3F).

Immunohistochemical detection of phage was performed essentially as follows. Tumor-bearing mice were injected intravenously via the tail vein with  $1 \times 10^9$  pfu CREKA (SEQ ID NO: 1) phage or non-recombinant T7 phage. After 15 minutes, the mice were perfused through the heart with 10 ml PBS. The indicated tissue was then dissected, fixed with 3.7% paraformaldehyde, and embedded in paraffin. Sections of 5  $\mu$ m were cut, de-paraffinized, washed with water, and treated with 3% hydrogen peroxide for 30 minutes. Rabbit anti-T7 antibody (Oku et al., Oncogene 21:2262-2269 (2002)) was diluted 1:1000 and applied, and the slides were incubated for one hour at room temperature. Slides were washed three times with PBS and then incubated with anti-rabbit IgG (DAKO) followed by incubation with 3,3'-diaminobenzidine (DAB; Sigma) according to the instructions of the manufacturer. The slides were washed with water and counter-stained with hematoxylin.



Peptide synthesis and labeling of peptides with fluorescein and rhodamine was carried out as described in Wender et al., Proc. Natl. Acad. Sci., USA 97:13003-13008 (2000), and Oku et al., *supra*, 2002, and detected

5 essentially as follows. A 1 mg/ml solution of fluorescein-labeled CREKA (SEQ ID NO: 1) peptide in PBS was injected into the tail vein of MMTV PyMT mice. After 15 minutes or two hours of circulation, mice were perfused through the heart with 10 ml PBS, and the

10 indicated organs harvested. Tissues were dissected, fixed with 3.7% paraformaldehyde for two hours, washed with PBS, and soaked in glycine (0.01%, pH 7.4) for 12 hours. Specimens were then treated with a sucrose gradient (12%, 15%, 18%), embedded in Tissue-Tek® (Miles

15 Inc.; Elkhardt, IN) and frozen. Subsequently, 5 µm sections were prepared for fluorescence microscopy, and 50 µm sections were prepared for confocal microscopy. To detect blood vessels, 100 µl of 1 mg/ml solution of fluorescein-labeled tomato lectin (Vector Labs;

20 Burlingame, CA) was injected into mice in PBS.

These results demonstrate that CREKA (SEQ ID NO: 1)-displaying phage or labeled CREKA (SEQ ID NO: 1) peptide quickly localizes to the vasculature of human or murine breast tumors in preference to the vasculature of

25 normal organs.

## EXAMPLE 3

A COLLAGEN IV ALPHA-2 CHAIN-RELATED PROTEIN BINDS TO THE  
CREKA (SEQ ID NO: 1) PEPTIDE

This example describes identification of a  
5 receptor for the CREKA (SEQ ID NO: 1) tumor homing  
peptide.

A. Identification of a receptor for the CREKA (SEQ ID  
NO: 1) peptide

To identify a receptor for CREKA (SEQ ID NO: 1)  
10 in breast tumor vasculature, a mouse breast cancer cDNA  
library was screened for binding of expressed proteins to  
immobilized CREKA (SEQ ID NO: 1) peptide. Among the  
individual phage clones obtained, one clone avidly bound  
to the peptide-coated surface (Figure 4A), but not to an  
15 uncoated surface treated with the blocking buffer only.  
Sequence analysis showed that this clone encodes a 138  
amino acid fragment related to the collagen IV alpha-2  
chain (see Figure 4B). The presence of Gly-X-Y repeats  
revealed that the fragment was derived from the triple  
20 helical portion of collagen IV.

Expression cloning was performed essentially as  
described in Essler and Ruoslahti, Proc. Natl. Acad.  
Sci., USA 99:2252-2257 (2002). CREKA (SEQ ID NO: 1)  
peptide was synthesized on a Symphony synthesizer (Rainin  
25 Instruments; Emeryville, CA) at The Burnham Institute  
peptide facility, and purified by high performance liquid  
chromatography. The peptide showed the correct mass by  
MALDI-TOF mass spectroscopy and was greater than 95%

pure. Biotin-labeled peptide (2 mg/ml) was immobilized on a streptavidin-coated 96 well Reacti-Bind® polystyrene strip plate (Pierce; Rockford, IL) by coating overnight at room temperature with 100 µl of 2 mg/ml biotin-CREKA  
5 (SEQ ID NO: 1) in PBS. The wells were subsequently treated three times with 200 µl SuperBlock® blocking buffer (Pierce).

A mouse breast carcinoma cDNA library from 4T1  
10 cells obtained from the American Type Culture Collection (ATCC) was prepared as follows. RNA from 4T1 cells was purified using a RNeasy® kit (Qiagen; Valencia, CA), and mRNA was twice purified from the RNA using an Oligotex™ Direct mRNA kit (Qiagen). Random-primed cDNA synthesis  
15 was performed using an OrientExpress™ cDNA synthesis kit (Novagen). The cDNAs were ligated into a modified T7Select1-2b phage vector (Novagen) between the 3' end of the T7 10B coat protein gene and the 5' end of nucleic acid sequence encoding a myc epitope in all three reading  
20 frames. The phage vector ligation products were packaged using a commercial packaging extract (Novagen) and amplified by infecting a BLT 5615 bacterial culture. The library diversity was  $1.2 \times 10^8$  as determined by a phage colony plaque assay. Phage clones that contained cDNA  
25 inserts without stop codons were isolated by sorting with monoclonal anti-myc antibodies (Chemicon; Temecula, CA) coated onto magnetic beads (Miltenyi Biotec; Auburn, CA). Phage clones that bound to the anti-myc antibody coated beads were recovered and amplified by infecting a BLT  
30 5615 bacterial culture. Three rounds of myc antibody sorting were performed. Over 90% of the phage clones from a random sampling were found to contain open reading

frame cDNA inserts, and the phage clones expressed, on average, protein fragments that were 75 amino acids in length.

Binding assays were performed as follows.

5 Phage suspension (100  $\mu$ l,  $10^6$  pfu/ $\mu$ l in PBS) was incubated in the 96 well Reacti-Bind<sup>®</sup> polystyrene strip plate for one hour, and the wells subsequently washed 20 times with 100  $\mu$ l PBS. Phage bound to immobilized CREKA (SEQ ID NO: 1) peptide were eluted with an excess of  
10 cognate SEQ ID NO: 1 peptide by application of 100  $\mu$ l of a 5 mg/ml solution of peptide SEQ ID NO: 1 in PBS. The phage were then recovered and amplified by infecting a BLT 5615 bacterial culture for 10 minutes at room temperature. Recovered phage were individually assayed  
15 for specific binding to CREKA (SEQ ID NO: 1) coated wells.

B. Binding of CREKA (SEQ ID NO: 1) to collagen is specific and is enhanced by denaturation

As shown in Figure 5A, the observed interaction  
20 between CREKA (SEQ ID NO: 1) and the collagen IV fragment SEQ ID NO: 2 was specific, as it could be inhibited by an excess of cognate CREKA (SEQ ID NO: 1) peptide in a dose dependent manner. Furthermore, as shown in Figure 5B, rabbit anti-mouse collagen IV serum, but not control  
25 serum, blocked the interaction between the phage-displayed collagen fragment and immobilized CREKA (SEQ ID NO: 1). Fibronectin, which binds to various collagens in their non-triple helical form, did not significantly reduce binding of the CREKA (SEQ ID

NO: 1)-displaying phage (Figure 5B; see Engvall et al.,  
*supra*, 1982).

CREKA (SEQ ID NO: 1)-displaying phage also were  
assayed for the ability to bind surfaces coated with  
5 collagens I, II, IV and X. As shown in Figure 5C, the  
CREKA (SEQ ID NO: 1) phage bound to each of these  
collagens. Furthermore, denaturation of the collagens by  
boiling enhanced the CREKA (SEQ ID NO: 1)-phage binding,  
indicating that non-helical collagen can be a receptor  
10 for CREKA (SEQ ID NO: 1) in tumor vasculature. In  
agreement with *in vivo* binding of CREKA (SEQ ID NO: 1) to  
collagen, co-injection of gelatin, which is denatured  
collagen I, completely blocked *in vivo* tumor homing of  
CREKA (SEQ ID NO: 1)-expressing phage.

15 For competitions with free CREKA (SEQ ID NO: 1)  
peptide, phage displaying the clone #3 collagen IV  
fragment (SEQ ID NO: 2;  $1 \times 10^7$  pfu) were applied to  
CREKA (SEQ ID NO: 1)-coated surfaces in 96-well ELISA  
plates prepared as described above, and incubated for one  
20 hour in the presence of 0.01 mg, 0.05 mg, or 0.1 mg  
soluble CREKA (SEQ ID NO: 1) peptide in 100  $\mu$ l PBS. In  
some experiments, phage were incubated with 100  $\mu$ g/ml  
fibronectin (Roche; Germany); with rabbit  
anti-collagen IV serum diluted 1:30 in PBS with 1 mM  
25  $MgCl_2$  and  $CaCl_2$ ; or with the same dilution of normal  
rabbit serum. After 20 washes with 100  $\mu$ l PBS, phage  
particles were recovered by infecting BL21 bacteria, and  
the number of attached phage determined. Phage output  
was determined as a percentage of phage recovered from  
30 wells not treated with soluble peptide.

To test phage binding to immobilized collagen, 96 well ELISA plates were treated for 12 hours at room temperature with native or boiled collagen I from calf skin (Sigma), native or boiled collagen II from chicken cartilage (Sigma), native or boiled collagen IV from Engelbreth Holm Swarm tumors (Sigma) or native or boiled collagen X from human placenta (Sigma), each at 200 µg/ml in PBS. The plates were blocked with 200 µl of SuperBlock® blocking buffer. After washing of the plates, CREKA (SEQ ID NO: 1) phage ( $1 \times 10^5$  pfu) were applied in each well and incubated for one hour. After washing with PBS, phage particles were recovered by infecting BL21 bacteria, and the number of pfu was determined as described above.

15

C. Localization of CREKA (SEQ ID NO: 1) peptide and collagen IV

Localization of collagen IV was compared to the localization of CREKA (SEQ ID NO: 1) peptide in breast tumors. As shown in Figure 6, FITC-CREKA (SEQ ID NO: 1) partially co-localized with collagen IV in connective tissue surrounding individual tumor masses in MMTV-PyMT mice. A FITC-labeled control peptide did not co-localize with collagen IV in MMTV-PyMT tumors. These results are consistent with a role for collagen IV as a receptor for the CREKA (SEQ ID NO: 1) peptide in tumor vasculature.

Immunostaining was performed after injection of 100 µg FITC-CREKA (SEQ ID NO: 1) into the tail vein of MMTV PyMT mice. After 15 minutes, tumors were dissected, fixed, stained for collagen IV and counter-stained with

DAPI essentially as follows. Collagen IV was detected by immunofluorescence as described in Engvall et al., *supra*, 1982, with the following modifications. Briefly, mouse tissues were fixed with 3.7% paraformaldehyde for two  
5 hours, washed with PBS, and incubated in glycine (0.01%, pH 7.4) for 12 hours to quench autofluorescence of the samples. Specimens were then treated with increasing concentrations of sucrose (12%, 15%, 18%), embedded in Tissue-Tek® (Miles Inc.), frozen, and cut into 5  $\mu$ m  
10 sections. After an overnight incubation with rabbit antiserum to collagen IV (diluted 1:40 in PBS) at 4°C (Engvall et al., *supra*, 1982), the sections were washed with PBS and incubated with rhodamine-labeled goat anti-rabbit antibody (DAKO) at a dilution of 1:30 for 30  
15 minutes. After washing, the sections were mounted in 90% glycerol.

In sum, the results described in this example demonstrate that the CREKA (SEQ ID NO: 1) peptide binds a collagen IV  $\alpha$ -2 chain related protein and further  
20 indicate that collagen IV or a related collagen can act as a receptor for tumor homing molecules in tumor vasculature.

All journal article, reference and patent citations provided above, in parentheses or otherwise,  
25 whether previously stated or not, are incorporated herein by reference in their entirety.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly,  
5 the invention is limited only by the claims.